GENTAMICIN INDUCED INTRACELLULAR TOXICITY IN *SACCHAROMYCES CEREVISIAE*

Lin Lin

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Mark G. Goebl, Ph.D., Chair

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Maureen A. Harrington, Ph.D.

Doctoral Committee

_____

Margaret E. Bauer, Ph.D.

Oct 25, 2010

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Mark C. Wagner, Ph.D.

_____

Bruce A. Molitoris, M.D.

DEDICATION

I dedicate my dissertation to my family, for their endless love, support and encouragement.

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ABSTRACT

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GENTAMICIN INDUCED INTRACELLULAR TOXICITY IN *SACCHAROMYCES CEREVISIAE*

At the present time, gentamicin is used in the treatment of both Gram-negative and Gram-positive bacterial infections. However, the poorly understood side effect of nephrotoxicity is a serious problem and is one of the dose-limiting factors in the use of gentamicin. In our model system, *Saccharomyces cerevisiae*, which is relatively resistant to gentamicin, at least 20 genes are required for gentamicin resistance. Inspection of the physical and genetic interactions of the gentamicin sensitive mutants reveals a network centered on the ARF pathway which plays a key role in the regulation of retrograde trafficking. Our studies show that *arf1*^{ts} *arf1*Δ *arf2*Δ cells, *gea1*^{ts} *gea1*Δ *gea2*Δ cells, and g*cs1*^{ts} *gcs1*Δ *glo3*Δ cells are all hypersensitive to gentamicin which indicates that impaired Arf1 function causes yeast cells to become hypersensitive to gentamicin. As evidence, cellular CPY trafficking and processing are blocked by the presence of gentamicin in some of these mutants. Interestingly, gentamicin can directly affect the level of the GTP-bound form of Arf1 in a cell growth phase-dependent manner; even though total Arf1 levels in *S. cerevisiae* are not affected. As predicted, we also find that gentamicin-bound resin can enrich both yeast Arf1-TAP protein and rat Arf1 protein *in vitro*. With the help of mass spectrometry, we also generated a gentamicin-binding

protein list. Gentamicin hypersensitivity is also observed in *S. cerevisiae* double deletion strains that lack both *ARF1* and *ARF2* but are kept alive by the presence of *hARF4* or *bARF1*. Increased -1 programmed ribosomal frameshifting efficiency is also observed in cells treated with gentamicin. Finally, a comparison of a gentamicin mixture and four of the gentamicin congeners reveals that gentamicin C1 is less toxic than other gentamicin congeners or the gentamicin total mixture.

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ABBREVIATIONS

AP	Adaptor protein
ARF	ADP-ribosylation factor
BFA	Brefeldin A
CCV	Clathrin-coated vesicle
CF	Cystic fibrosis
CFTR regulator	Cystic fibrosis transmembrane conductance
CGN	*cis*-Golgi network
COPI	Coat protein complex I
COPII	Coat protein complex II
CPY	Carboxypeptidase Y
DMD	Duchenne muscular dystrophy
eEF-1 complex	Eukaryotic elongation factor-1 complex
ER	Endoplasmic reticulum
GAP	GTPase Activating Protein
GARP	Golgi-associated retrograde protein complex
GEF	Guanine Nucleotide Exchange Factor
Gga proteins	γ-ear adaptin homology Golgi associated Arf-binding proteins
HOPS	Homotypic fusion and vacuole protein sorting
LC-MS	Liquid chromatography-mass spectrometry
NMD	Nonsense-mediated mRNA decay pathway
ORF	Open reading frame

PRF	Programmed Ribosomal Frameshifting
RAC	Ribosome-associated chaperone complex
SGD	*Saccharomyces* genome database
TAP	Tandem affinity purification
TGN	*trans*-Golgi network

INTRODUCTION

The discovery of the first aminoglycoside antibiotic, streptomycin, was a landmark in the history of antibiotics in 1943. Streptomycin was the first useful antibiotic isolated from a bacterial source and the first effective therapeutic agent against *Mycobacterium tuberculosis*, for which penicillin was largely ineffective (Schatz, Bufie, & Waksman, 1944).

The success of streptomycin led to the subsequent isolation of a large number of other aminoglycosides from a variety of producers. Among them, gentamicin, isolated from *Micromonospora purpureochromogenes* in 1963, constituted a significant advance in the treatment of both Gram-negative and Gram-positive bacterial infections (Weinstein et al., 1963). In many cases, gentamicin was the only effective therapeutic tool against multidrug-resistant bacterial strains (Appel & Neu, 1978). At the present time, gentamicin is the most widely used aminoglycoside and remains a mainstay of treatment of life-threatening Gram-negative bacterial infections, as it is cheap and widely available.

However, the use of gentamicin is limited by its associated toxicity, involving the renal system and the auditory system. Although once-daily dosage regimens and effective monitoring procedures have definitely improved the situation, our knowledge of safer gentamicin usage is still insufficient (Buijk et al., 2004). In this study, my goal was to expand our understanding of gentamicin cytotoxicity.

1 Chemical Structure of Gentamicin

Commercially available gentamicin sulfate salt is not a single compound but a mixture of four active congeners, gentamicin sulfate salt C_1, C_{1a}, C_2 and C_{2a} (British Pharmacopoeia, Volumes I and II, 2008) (Figure 1). These different congeners have

different patterns of methylation modification at the 6' position of ring I. Cobalt was demonstrated to stimulate the two C-methylation steps at gentamicin biosynthesis (Tipson & Horton, 1978). Thus, the biosynthesis of gentamicin C_2 and gentamicin C_1, but not C_{1a} or C_{2b}, are cobalt dependent (Kumar, 2008). All the congeners appear to have similar antimicrobial activity (Weinstein, Wagman, Oden, & Marquez, 1967).

Gentamicin sulfate salts occur as white to buff powders and are soluble in water. The amine groups of the gentamicin sugar rings exhibit variable p*Ka* values that range from 5.6 to 9.5. The amine groups on ring I are the most basic (p*Ka* ~ 9.6), whereas those on ring II (p*Ka* ~ 5.6 to ~ 8.0) and ring III (p*Ka* ~ 7.5) have *pKa* values closer to physiological pH. Gentamicin thus carries a net positive charge under physiological conditions (pH 7.4) (Wilson, Richard, & Hughes, 1973; Arya, 2007).

2 Clinical Uses and Side Effects of Gentamicin

During the first twenty years after its discovery, gentamicin was used extensively (Black, Calesnick, Williams, & Weinstein, 1963) mainly because of its antimicrobial efficacy at relatively low serum concentration, few resistant organisms, and a relatively low prevalence of clinical toxicity (Appel & Neu, 1978). Currently, it is often used clinically in combination with other antimicrobial agents such as β-lactams or glycopeptides for the treatment of serious infections with Gram-negative and Gram-positive organisms.

Gentamicin is effective against a broad spectrum of both Gram-positive and Gram-negative organisms. Among the responsive Gram-positive groups of microorganisms are *Staphylococcus aureus* (including many penicillin-resistant strains) and *Streptococcus pyogenes*. Among the clinically more important species of Gram-

negative organisms responsive to gentamicin are *Proteus mirabilis, Pseudomonas aeruginosa*, *Escherichia coli*, *Enterobacter cloacae*, *Klebsiella pneumoniae*, *Salmonella typhimurium*, and *Shigella* (Black, Calesnick, Williams, & Weinstein, 1963).

Gentamicin is a low protein-binding drug (<10%) with a poor degree of oral absorption, thus intravenous administration is the usual route in patients with severe infections. After intravenous administrations, the concentration of gentamicin in secretions and most tissues is low. However, high concentrations are found in renal cortical tissue and the hair cells of the inner ear.

After it is freely filtered through the glomerulus in the kidney without being metabolized, most of the intravenously administered gentamicin is excreted into the urine. But about 10% of the dose accumulates in the renal cortex where ultrafiltration occurs; thus, the gentamicin concentration in the renal cortex can be several times higher than in the serum (reviewed in Chambers, 2001). Gentamicin could also remain in the renal cortex due to its long half-life there (100 hours comparing to 30 minutes in the plasma), which makes renal damage worse (Luft, 2002). Molecular level studies showed that, following glomerular filtration, gentamicin can diffuse from the tubular lumen to the apical membranes of renal proximal tubular cells (reviewed in Schrier, 1999). By binding to the negatively charged phospholipid-binding sites on the brush border of the apical membranes, gentamicin enters the cell mainly by megalin-mediated endocytosis. Megalin is an endocytic receptor protein which is most abundantly expressed in the renal proximal tubular cells (Moestrup et al., 1995). A small but toxicologically important portion of the administered gentamicin is then accumulated in the proximal tubule cells. Gentamicin-induced nephrotoxicity is characterized by tubular necrosis which mainly

involves the proximal tubule cells, a slow rise in serum creatinine, and a marked decrease in the glomerular filtration rate (GFR).

Unlike gentamicin-induced nephrotoxicity, gentamicin-induced ototoxicity is more related to the peak serum level of gentamicin during treatment (reviewed in Selimoglu, 2007). Gentamicin can accumulate in the endolymph and perilymph of the inner ear and injures hair cells also by megalin-mediated endocytosis. Some mutations on the human mitochondrial 12S ribosomal RNA gene have been previously reported to predispose carriers to gentamicin-induced ototoxicity (reviewed in Fischel-Ghodsian, 2005). Thus, the prevention of gentamicin-induced ototoxicity through family history and molecular diagnosis is possible in many cases.

Since an accumulation from multiple doses or a high serum concentration of gentamicin can lead to nephrotoxicity and ototoxicity, clinicians proposed that the serum level of gentamicin should always be carefully monitored (Bennett et al., 1979). Traditionally, gentamicin was administered 2-3 times daily. As an improvement, recent insight into the pharmacodynamics of gentamicin has suggested once-daily dosing regimens which include all the 2-3 separated daily dosage. Clinicians have demonstrated the efficacy, safety, and economy of the 24-hour dosing interval, resulting in it becoming the standard for gentamicin administration (Nicolau et al., 1995; Barza et al., 1996; Ward & Theiler, 2009).

However, gentamicin still induces a dose-dependent nephrotoxicity in 10-25% of therapeutic courses, despite rigorous monitoring of serum drug concentration and once-daily dosing regimens (Hatala et al., 1996).

The differences in chemical structures among the gentamicin congeners are similar to the differences between gentamicin and some other aminoglycosides, such as tobramycin or netilmicin (United States Pharmacopoeial Convention, 1995). It was reasonable to assume that even though all the major gentamicin congeners, C_1, C_{1a}, C_2 and C_{2a}, appear to have similar antimicrobial activity (Weinstein et al., 1967), they exhibit different cellular toxicity and nephrotoxicity *in vivo* (Sandoval et al., 2006). Among them, congener C_1 (previously identified as C_2) exhibited the least cellular toxicity and no nephrotoxicity (Sandoval et al., 2006).

3 Mechanism of Gentamicin Antimicrobial Activity

Since their discovery, the biochemical modes of action of aminoglycosides have long been topics of great interest. A series of genetic and biochemical studies in the late 1950s and early 1960s proposed the ribosome as the probable target for the antibacterial actions of many aminoglycosides (Erdos & Ullmann, 1959). But the ribosome exceeds the size of an average aminoglycoside by four orders of magnitude and presents multiple sites for aminoglycoside binding. It was only after the advent of ribosome crystallography that detailed views of the ribosome-gentamicin interactions at atomic resolution were revealed (reviewed in Tenson & Mankin, 2006).

The bacterial ribosomal small subunit, which is called the 30S subunit, contains an RNA chain (16S) and 20-21 proteins. When elongation proceeds, the small subunit provides the decoding center, which possesses three tRNA-binding sites, A (aminoacyl), P (peptidyl) and E (exit) sites. Decoding requires the correct codon-anticodon recognition and thus controls translation fidelity.

Gentamicin is positively charged at biological pH, which contributes to its rRNA binding. Gentamicin-binding affects ribosome accuracy at the initial step of aminoacyl-tRNA selection. Ribosome crystallography reveals that when there is no gentamicin around, two conserved adenine residues on the decoding center aminoacyl-tRNA site (A site) of 16S rRNA at positions 1492 and 1493 (*E. coli* numbering) (Yoshizawa et al., 1998; Ogle et al., 2001) always stack in the internal loop of helix 44. The binding of mRNA and cognate tRNA in the A site induces A1492 and A1493 to flip out of the internal loop of helix 44 and to issue an approval signal from the decoding center. In the presence of bound gentamicin in helix 44, conformation changes happen in the decoding center. As a result, A1492 and A1493 flip out and always stay out of the internal loop of helix 44, even upon the binding of a near-cognate tRNA. During translation initiation, gentamicin interferes with the binding of fMet-tRNA and the formation of the initiation complex. During translation elongation, gentamicin increases the frequency of amino acid misreading and the bacteria die because they are unable to synthesize proteins vital to its growth.

Though chemically distinct, aminoglycoside antibiotics that contain ring I and II all bind in the same RNA pocket through similar hydrogen bonding networks. Additional rings (like ring III of gentamicin) contribute to binding affinity and assist in the correct orientation of rings I and II by creating additional drug-RNA contacts (Tenson & Mankin, 2006).

Gentamicin congeners bind to the 16S rRNA at the same binding site but with different affinities (Yoshizawa et al., 1998). Gentamicin C_{1a} binds to 16S rRNA with a

slightly higher affinity (K_d = 0.01 μM) than C_2 (K_d = 0.025 μM), whereas C_1 binds with the lowest affinity compared to the others (K_d = 0.5 μM).

Although the eukaryotic ribosome is much more resistant to aminoglycosides, there is similarity between prokaryotic and eukaryotic protein synthetic machinery. Aminoglycoside antibiotics bind to the 18*S* rRNA in the eukaryotic ribosomal small subunit but, compared to the binding to the prokaryotic 16*S* rRNAs, with a 1-2 orders of magnitude lower binding affinity (Fan-Minogure, 2007; Recht et al., 1999).

4 Effects of Gentamicin on Eukaryotic Translation Fidelity

4.1 Suppressing nonsense mutations

Interestingly, over twenty years ago, clinicians found that cystic fibrosis (CF) patients, who commonly require gentamicin as an antibiotic to treat *P. aeruginosa* upper respiratory infections by both intravenous administration and inhalation, were able to tolerate the drug and experienced less nephrotoxicity (Stephens et al., 1983; McCracken, 1986). Further investigation elucidated that cystic fibrosis is caused by mutations in the gene encoding the cystic fibrosis transmembrane conductance regulator (CFTR) protein. Among all the mutations, the *CFTR* mutations generating premature stop codons lead to nonsense-mediated *CFTR* mRNA decay and result in a very small amount of protein product (reviewed in Kerem, 2004).

Bedwell's laboratory first reported in their transgenic mouse model, which expresses a human *CFTR* mutant (CFTR-G542X) in an animal lacking endogenous mouse *CFTR* (*Cftr*$^{-/-}$), that gentamicin can suppress the premature termination of *CFTR*. Later, they also reported that gentamicin can increase the abundance of *CFTR* mRNA and

partially restore the expression of functional CFTR protein in both human cell lines and their transgenic mouse model (Howard et al., 1996; Du et al., 2002; Du et al., 2006).

At the molecular level, studies showed that gentamicin can suppress premature stop codon mutations by disrupting translational fidelity. By allowing the incorporation of near-cognate tRNA at the premature codon on transcripts, gentamicin helps translation to continue until the normal stop codon in the transcript is reached (Sermet-Gaudelus, 2007). Gentamicin also prevents nonsense-mediated mRNA decay since the ribosome can translate through the premature termination codon. However, the susceptibility to suppression depends on the premature stop codon itself and on the sequence context surrounding the premature stop codon (Phillips-Jones et al., 1995).

Nevertheless, the ability of gentamicin to correct other nonsense mutation diseases, like Duchenne Muscular Dystrophy (DMD), *in vivo* has been reported (Barton-Davis, 1999).

Currently, gentamicin and other aminoglycosides are widely considered potential therapeutics for the treatment of many diseases resulting from nonsense mutations (Phase III Clinical trials gov identifier: NCT00803205, Study of Ataluren (PTC124™) in Cystic Fibrosis; Phase I Clinical trials gov identifier: NCT00451074, Six Month Study of Gentamicin in Duchenne Muscular Dystrophy with Stop Codons). Gentamicin may also prove to be beneficial in cancers, such as colon cancer, that result from nonsense mutations in tumor suppressor genes (Kaufman, 1999).

4.2 Programmed ribosomal frameshifting

Despite rigorously maintaining the reading frame of mRNA molecules to be translated into proteins, ribosomes also make programmed slips by one base towards

either the 5'(-1) or the 3' (+1) direction during translation (Jacks & Varmus, 1985). This programmed ribosomal frameshifting is controlled by frameshifting signals, including special sequence information and structural elements in mRNA molecules (Farabaugh, 1996; Harger et al., 2002). Viruses use this mechanism to increase the protein-coding capacities of their genomes, whose sizes are limited due to the small volumes of viral capsids into which they are packaged (Farabaugh, 1996). Programmed frameshifting was first discovered in viruses, but it has also been observed in prokaryotic and eukaryotic systems (Jacks & Varmus, 1985; Muldoon-Jacobs & Dinman, 2006; Lux et al., 2010).

The yeast *S. cerevisiae* has proven to be an ideal model system to study programmed ribosomal frameshifting. Researchers have observed that the deletion of any member of the conserved nascent peptide-associated complex, especially the deletion of *SSZ1*, *ZUO1*, or *SSB1*/*SSB2*, can inhibit -1 programmed ribosomal frameshifting but has no effect on +1 programmed ribosomal frameshifting (Muldoon-Jacobs & Dinman, 2006).

Our goal was to clarify the effect of gentamicin on programmed ribosomal frameshifting efficiency since it has not been reported in the literature. Recently, an *in vivo* dual-luciferase assay was developed to improve the previous *lacZ*-luciferase bicistronic reporter system (Harger & Dinman, 2003) (Figure 2). The frameshifting signals in the new dual-luciferase assay are inserted between the *Renilla* and firefly luciferase reporter genes, and the two activities can be directly measured in cell lysates in a single tube.

5 Pathway for the Uptaking and Trafficking of Gentamicin

5.1 Endocytic pathway and retrograded trafficking pathway

The complete picture of how gentamicin induces nephrotoxicity involves multiple intracellular mechanisms. Gentamicin is taken up through the endocytic pathway like other cellular toxins such as cholera. Then, gentamicin is trafficked in a retrograde fashion through the Golgi apparatus to the endoplasmic reticulum, from where it is finally released into the cytosol (Kahn et al., 1991; Moss & Vaughan, 1995). It has been shown that gentamicin sequestered in the vacuoles or trafficked in a retrograde fashion through the secretory pathway is nontoxic; however, the gentamicin released into the cytosol is toxic (Sandoval & Molitoris, 2004).

5.2 Cellular toxicity induced by an impaired intracellular trafficking pathway

Previous research in the Molitoris' laboratory (Indiana University School of Medicine) has shown that gentamicin accumulated by proximal tubule cells is mainly localized within endosomes, lysosomal vacuoles, and the Golgi apparatus (Sandoval et al., 1998; Sandoval et al., 2000). Because gentamicin is positively charged in endosomes (pH = 5.9~6) and lysosomes (pH = 4.8~5), it is believed that gentamicin is unable to pass across the membranes and act outside of endosomes or lysosomes. But, when lysosome permeability is changed, gentamicin is released into the cytosol more quickly and gentamicin-induced cellular toxicity can be severe (Sandoval & Molitoris, 2004; Servais et al., 2005).

In the model system Bakers yeast *S. cerevisiae*, further investigation revealed that the deletion of any of the four genes encoding the protein components of the Golgi-associated retrograde protein (GARP) complex causes sensitivity to gentamicin (Wagner

et al., 2006). GARP is a membrane-tethering complex that facilitates retrograde transport of endosome vesicles which can then fuse into the *trans*-Golgi network. Defects in the GARP complex may cause defects in retrograde trafficking from endosomes back to the Golgi and lead to vacuole fragmentation (Conibear et al., 2003). Blackburn and Avery found that cells lacking Vps54 (Luv1), a component of the GARP complex, have increased sensitivity to gentamicin (Blackburn & Avery, 2003). Our laboratory further demonstrated that cells lacking other components of the GARP complex, Vps51, Vps52, or Vps53, are also hypersensitive to gentamicin (Wagner et al., 2006). In the GARP complex, Vps52, Vps53 and Vps54 form a complex in a 1:1:1 ratio (Conibear & Stevens, 2000). If one of these proteins is missing, the other two proteins become unstable. Vps51 is assembled with Vps52, Vps53 and Vps54 and also binds the t-SNARE protein Tlg1, which is in close proximity to the t-SNARE protein Tlg2 (Piper et al., 1994). Vps45 is a key regulator of the GARP pathway (Bryant & James, 2001). The absence of Vps45 results in strong gentamicin hypersensitivity also (Wagner et al., 2006). Vps45 is a member of the Sec1/Munc-18 (SM) family, whose members have essential roles in regulating multiple membrane transport pathways. When yeast cells lack the SM protein Vps45, the t-SNARE Tlg2 can still be targeted to the correct intracellular location. However, Tlg2 is non-functional and unable to bind its cognate SNARE binding partners Tlg1 and Vti1. Vps45 acts as a chaperone-like molecule for its cognate t-SNAREs and plays an essential role in the activation process, allowing its cognate t-SNAREs to participate in ternary complex formation (Bryant & James, 2001).

The yeast counterpart of the mammalian lysosome is the vacuole. the Goebl laboratory has also tested strains that have deletions of different genes encoding proteins

that are components of the homotypic fusion and vacuole protein sorting (HOPS) complex (Wagner et al., 2006). The HOPS complex, consisting of Pep3, Pep5, Vam6, Vps16, Vps33, and Vps41, is critical for proper tethering of endosomal vesicles to the vacuolar membrane (Seals et al., 2000). Mutants lacking Vps16 or Vps41 of the HOPS complex have increased sensitivity to gentamicin. Cells lacking the HOPS complex associated protein Vps8 also have increased sensitivity to gentamicin. The HOPS complex acts as a guanine nucleotide exchange factor (GEF) for the Rab-like protein Ypt7. The HOPS complex facilitates nucleotide exchange on Ypt7. Thus the HOPS complex functions as a Ypt7 effector. In yeast, vacuole SNARE complexes bind to either Sec17 or the HOPS complex, but not both (Collins et al., 2005). Sec17 and its co-chaperone Sec18 disassemble SNARE complexes. Ypt7 regulates the reassembly of unpaired SNAREs with each other and with the HOPS complex, forming HOPS-SNARE complexes prior to fusion. Thus, Sec17 and Ypt7-regulated HOPS have mutually exclusive interactions with vacuole SNAREs. They mediate SNARE complex disruption and assembly for docking and fusion respectively.

5.3 Genome-wide screening for gentamicin sensitive strains

To identify genes that are required for gentamicin resistance in the yeast *S. cerevisiae*, Blackburn and Avery tested a library of the yeast *S. cerevisiae*, which contained defined deletions of each single nonessential yeast gene, for gentamicin hypersensitivity (Blackburn & Avery, 2003). Among approximately 4,800 defined deletion strains, seventeen mutants are hypersensitive to gentamicin as compared to the wild-type parent strain. Genome-wide screening was carried out on the yeast extract-peptone-dextrose (YPD) medium plates either in the absence or presence of gentamicin.

When the seventeen deletion strains were moved to YPD liquid medium, only some of them showed growth inhibition in the presence of gentamicin (Wagner et al., 2006). The deletion strains with clear growth inhibition in the presence of gentamicin were: *vps54*Δ, *vps16*Δ, *zuo1*Δ, *vps15*Δ, *sac1*Δ, *gcs1*Δ, and *ydr455c*Δ (the dubious open reading frame which partially overlaps with *NHX1*). The gene products Vps54 (a member of the GARP complex) and Vps16 (a member of the HOPS complex) were discussed in the previous section.

Zuo1 is a cytosolic ribosome-associated chaperone which contains a dnaJ domain and functions as a J-protein partner (Conz et al., 2007; Gautschi et al., 2001). The dnaJ/Hsp40 is a family of proteins that regulates Hsp70 chaperone activity. All dnaJ homologs contain a highly conserved 75-amino acid J domain, which interacts with the ATPase domain of Hsp70 to stimulate ATP hydrolysis. DnaJ homologs thus stimulate the intrinsically weak ATPase activity of Hsp70 proteins and facilitate the interaction between Hsp70 and polypeptide substrates (Huang et al., 2005). Along with the Hsp70-related protein Ssz1in the yeast *S. cerevisiae*, Zuo1 forms the ribosome-associated complex (RAC), which stimulates the ATPase activity of the Hsp70 proteins Ssb1 and Ssb2. Together, Zuo1, Ssb1/Ssb2 and Ssz1 act as a molecular chaperone complex to facilitate protein folding of nascent polypeptide chains. Mutations in *ZUO1*, *SSB1*/*SSB2* and *SSZ1* lead to improper translation termination and inhibition of -1 ribosome frameshifting (Muldoon-Jacobs & Dinman, 2006).

Vps15 is a serine/threonine protein kinase that functions with Vps34 to form a membrane-associated signal transduction complex. *VPS34* was also suggested to be required for gentamicin resistance in the yeast *S. cerevisiae* (Blackburn & Avery, 2003).

The Vps15/Vps34 complex is the only known phosphatidylinositol 3-kinase in the yeast *S. cerevisiae* (Herman & Emr, 1990). Vps15 and Vps34 are the regulatory subunit and the catalytic subunit of the complex, respectively. The recruitment of Gpa1, which is a mating specific protein, promotes the activation of the complex (Slessareva et al., 2006). Notably, both Vps15 and Vps34 are necessary for recruitment of Gpa1 to the endosome. Gpa1 is the GTP-binding α-subunit of a heterotrimeric G protein and can be activated by GEF proteins (Slessareva et al., 2006). Vps15 binds directly to the inactive (GDP-bound) form of Gpa1 while Vps34 directly engages the active (GTP-bound) form of Gpa1. When engaged to active Gpa1, Vps34 is stimulated from its basal activity and the production of phosphatidylinositol 3-phosphate is increased (Herman & Emr, 1990).

Sac1 is an integral membrane phosphatidylinositide phosphatase. Sac1 functions along with the phosphatidylinositol 4-kinases, Pik1, Stt4, and Lsb6 at Golgi membrane and the nuclear membrane, the plasma membrane, or both the vacuolar and the plasma membranes, respectively (Walch-Solimena & Novick, 1999; Cutler et al., 1997; Han et al., 2002). *SAC1* mutants accumulate PtdIns(4)P at the ER and vacuolar membranes, which in turn results in altered late endocytic and vacuolar trafficking (Tahirovic et al., 2005). Mutations in Stt4, but not in Pik1p or Lsb6p, compensate for this defect (Cutler et al., 1997). *SAC1* mutants also display defects in endosomal trafficking and synthetic interactions with mutations in a variety of genes involved in the vacuolar protein sorting pathway (Walch-Solimena & Novick, 1999).

Gcs1 is a GTPase activating protein (GAP) for ADP-ribosylation factors (Arfs, including Arf1, Arf2, and Arf3 in the yeast *S. cerevisiae*) (Blader et al., 1999). Arfs were originally identified as cofactors of cholera toxin and more recently recognized as

essential participants in intracellular vesicular transport (Kahn et al., 1991; Moss & Vaughan, 1995). Arfs are small GTPases and members of the Ras superfamily which can cycle between active-GTP-bound and inactive-GDP-bound conformations (Roth, 1999; Gillingham & Munro, 2007). Guanine nucleotide exchange factors (GEF) can activate Arfs to a GTP-bound conformation while GTPase activating proteins (GAP) can deactivate Arfs to a GDP-bound conformation. Sec7, Gea1 and Gea2, and Syt1 have been identified as Arf1 GEF proteins. The temperature sensitive *GEA* mutants were found to have defects in ER-to-Golgi and intra-Golgi transport (Peyroche et al., 1996). Gcs1, Glo3, and Age2 are Arf1 GAP proteins. Among them, Gcs1 and Age2, an essential Arf GAP pair, provide an overlapping function for vesicle transport from the trans-Golgi network to the vacuole and late endosomes. Gcs1 and Glo3, another essential Arf GAP pair, also provide an overlapping function for vesicle retrograde transport from the Golgi to the ER (Poon et al., 2001). Furthermore, a *gcs1*Δ strain was found to be weakly hypersensitive to gentamicin (Blackburn & Avery, 2003). Much like other GTPases, the Arfs do not efficiently hydrolyze GTP in the absence of an Arf1 GAP (Randazzo et al., 1994). These GEFs and GAPs play an important role in regulating Arf1 activity. I will explore the ARF1 signaling pathway in greater detail in my dissertation.

The *NHX1* gene is present on the opposite DNA strand but overlaps *YDR455C*. Nhx1 is an endosomal Na^+/H^+ exchanger. It has been well established that luminal acidification of the endocytic components, including endosomes and the lysosome/vacuole, is required for endosomal maturation, receptor recycling, and vesicle targeting (Brett et al., 2005). Loss of Nhx1 confers growth sensitivity to low pH stress and trafficking defects, which can be alleviated by weak bases (Bowers et al., 2000).

Previous studies have also shown that gentamicin can inhibit the Nhx1 Na^+/H^+-ATPase *in vivo* (Ali et al., 1995). Furthermore, these investigators demonstrated that gentamicin inhibits the Na^+/H^+-ATPase in renal tubule cells only when the Na^+/H^+-ATPase is accessible from the cytoplasm (Fukuda et al., 1991).

5.4 Targeting the Arf1 pathway

The Arfs are structurally and functionally conserved proteins of approximately 21 kDa, and are members of the Ras superfamily of regulatory GTPases (reviewed in Pucadyil & Schmid, 2009). In *S. cerevisiae*, three Arfs have been identified, Arf1, Arf2, and Arf3. Arf1 and Arf2 are 96% identical in amino acid sequence and are functionally interchangeable and form an essential pair of proteins (Stearns et al., 1990). Arf1 is an abundant protein, and the level of protein produced from *ARF1* is approximately 10-fold higher than that from *ARF2* (Stearns et al., 1990). While an *arf2*Δ strain displays no growth phenotype, *arf1*Δ strains grow slowly, are cold sensitive, and are fluoride hypersensitive. It is likely that the genetic differences between mutations in *ARF1* and *ARF2* are due to differences in the level of expression of the two proteins (Kahn et al., 1991; Moss & Vaughan, 1995). *ARF3* does not compensate for the loss of *ARF1* and *ARF2*, and an *arf1*Δ*arf2*Δ double deletion strain is inviable despite the presence of Arf3 (Stearns et al., 1990). Hereafter, I will focus on the ARF1 pathway signaling.

Based on genetic interactions between hypersensitive mutants and their products, we proposed that the ARF1 pathway itself is a target and is affected by gentamicin (Figure 3).

The ARF1 pathway plays a critical role in chitin synthesis in yeast *S. cerevisiae*. Chitin is a long-chain polymer of *N*-acetylglucosamine and it is a major cell wall

component (Trautwen et al., 2005). By controlling the sporulation-specific protein Sps1, the ARF1 pathway regulates the subcellular localization of the chitin synthase protein Chs3 (Iwamoto et al., 2005). *CHS1* encodes another chitin synthase whose subcellular localization is not regulated by Sps1 (Ziman et al., 1998) but is also required for gentamicin resistance (Blackburn & Avery, 2003). *CAX4* and *MNN9* encode glycosylation enzymes essential for cell wall synthesis (Ram et al., 1994) and are also required for gentamicin resistance (Blackburn & Avery, 2003).

The phosphatidylinositol 3, 4, 5-trisphosphate can activate Arf -nucleotide exchange factors through their Pleckstrin homology domain (PH domain) and their Sec7 domain directly and, in turn, up-regulate Arf1 activity (Klarlund et al., 1997). The phosphatidylinositol 3, 4, 5-trisphosphate is regulated by phosphatidylinositide phosphatase Sac1 and phosphatidylinositol kinases Vps15/Vps34 and Pik1 which are all required for gentamicin resistance (Blackburn & Avery, 2003).

As mentioned previously, both the HOPS and GARP complexes regulate Ypt/Rab's. The Arfs and Ypt/Rabs are two families of GTPases and are key regulators of vesicular transport. While Arfs are implicated in vesicle budding from the donor compartment, Ypt/Rab's are involved in the targeting of a vesicle to an acceptor compartment (Kawasaki, Nakayama, & Wakatsuki, 2005). Interestingly, several proteins that belong to the Sec7-domain family exhibit distinct genetic interactions with Ypt/Rab's (Jones et al., 1999) which lead to the proposal that an Ypt-Arf GTPase cascade exists in the secretory pathway.

The Arfs have an important role in regulating secretory membrane transport and modulating Golgi structure (reviewed in D'Souza-Schorey & Chavrier, 2006; Spang,

2002). Arf1 regulates the composition of secretory and endocytic organelles by recruiting the vesicle coat proteins COPI and clathrin in *S. cerevisiae*. Importantly, the recruiting of clathrin is through the clathrin-coated cargo adaptor proteins, AP-1, AP-2, AP-3, AP-4, Gga1 and Gga2 in *S. cerevisiae*. The Gga proteins are ubiquitous Golgi-localized adaptor proteins which can interact with Arf1 and Arf2 in a GTP-dependent manner (Puertollano et al., 2001). We will utilize the binding ability of Gga2 to Arf1-GTP in our experiments.

Although Arf1 is soluble when bound to GDP, it can associate with membranes because of the N-terminus myristoylation (covalent attachment of myristate, a 14-carbon saturate fatty acid, to the N-terminal glycine, [Franco et al., 1995]). *N*-Myristoylation promotes weak and reversible protein-membrane and protein-protein interactions. This lipid modification appears to be important for Arf1 association with membranes (Goldberg, 1998). Recent research showed that the myristoyl group inhibits the interaction of Arf1 with its nucleotide exchange elements, unless there is a membrane surface available which can accommodate the myristoyl chain. So nucleotide exchange can only happen after the association of the myristoyl group with a membrane surface (Liu et al., 2009).

Brefeldin A (BFA) has been a very useful tool for cell biologists studying the structure and function of intracellular organelles, particularly the Golgi apparatus of mammalian cells. The structure of the Golgi complex is severely perturbed after only a few minutes of BFA treatment. After ten minutes of exposure to the drug, the Golgi complex disassembles and fuses with the endoplasmic reticulum (ER) (Lippincott-Schwartz et al., 1989). In permeable strains of the yeast *S. cerevisiae*, similar BFA

induced changes in Golgi morphology can be observed (Peyroche et al., 1996). It was later established that Brefeldin A could stabilize the abortive Arf1-GDP-Sec7 domain complex (the Sec7 catalytic domain was from Gea1 in this study) and prevent Arf1 from being activated by Arf GEF through the Sec7 domain (Chardin & McCormick, 1999).

6 Binding of Gentamicin to Other Cytosolic Targets

The binding ability of gentamicin to some protein partners has been described. Horibe et al. (2004) applied a protein pull-down assay to a gentamicin-Sepharose column and analyzed the pull-down proteins by 1D SDS-PAGE (Horibe et al., 2004). Calreticulin (CRT, UniProtKB: P52193) was suggested to bind to gentamicin directly *in vitro* with a K_D value of 3.85×10^{-4} M. The binding of ribostamycin to the protein disulfide isomerase (PDI, UniProtKB: P05307) (with a K_D value of 3.19×10^{-4} M), the binding of gentamicin to the heat shock protein 90 kDa beta member (GRP94, UniProtKB: Q95M18) (without a K_D value), and heat shock protein 70 kDa (HSP73, UniProtKB: CAN13333 and CAN87708) (without a K_D value) were all elucidated through the same experimental strategy (Miyazaki et al., 2004; Horibe et al., 2001). However, it still remains unclear whether all the proteins that can bind to gentamicin have been identified.

One of the limitations of the previous experimental method is the poor resolution of one-dimensional SDS-PAGE, because only abundant proteins which form strong Coomassie Blue stained bands can be identified. During the past two decades, mass spectrometry has become established as the primary method for protein identification from complex mixtures of biological origin. With the power of mass spectrometry, we

reasoned that less abundant gentamicin binding proteins in pull-down mixtures could be identified.

7 Other Intracellular Toxicity Hypotheses

A few more hypotheses have been suggested for the mechanisms of gentamicin intracellular toxicity, including induction of hydroxyl radical mediated tissue injuries, apoptosis through the mitochondrial pathway, and increased renal cortical phospholipidosis.

Gentamicin was found to form complexes with mitochondrial Fe^{2+} to catalyze the formation of superoxide anions, hydrogen peroxide, hydroxyl radicals, and water (Walker & Shah, 1988). Hydroxyl radicals are strong mediators of tissue injury. Hydroxyl radicals can oxidize a wide variety of organic compounds including polyunsaturated fatty acids, leading to cell membrane injury and protein degradation (Beckman et al., 1990). Hydroxyl radicals also play a role in toxic acute renal failure (reviewed in Baliga et al., 1999). Notably, scavengers of reactive oxygen metabolites as well as iron chelators provide protection from gentamicin-induced nephrotoxicity (Paller et al., 1984).

Gentamicin can also increase caspase-9 and caspase-3 activities and, in turn, activate apoptosis in renal cell lines and embryonic rat fibroblasts (Mouedden et al., 2000) through the mitochondrial apoptosis pathway (Servais et al., 2004).

The binding of gentamicin to phosphatidylinositol, which lies in lipid bilayers, can cause lipid aggregation (Bambeke et al., 1995) and inhibit the activities of phospholipases (Hostetler & Hall, 1982). Although neither phospholipid accumulation nor phospholipase inhibition alone can explain cell death, the extent of phopholipidosis

induced by most aminoglycosides correlates well with their nephrotoxic potential (Laurent et al., 1982).

8 Research Objectives

The overall objective of this research project is to expand our understanding of the mechanisms of gentamicin cytotoxicity. However, this study is difficult in mammalian cells due to their inherent complexity. Therefore, given the tremendous genetic, genomic and proteomic advantages of yeast, and demonstrated similarities between yeast and mammalian cells, we have begun to utilize yeast to understand the pathway(s) important in inducing gentamicin toxicity. Specifically, Aim 1 and Aim 4 of this study are centered on the ARF1 pathway which plays a key role in the regulation of retrograde trafficking from the Golgi-to-the ER *in vivo*. In brief, four specific aims are addressed to achieve this task.

Aim 1 was to elucidate the role of the Arf1 pathway in gentamicin toxicity. Since we hypothesize that the Arf1 pathway is one of the intracellular targets of gentamicin, we examined whether *ARF1*, *ARF1*-GEF, or *ARF1*-GAP mutant strains are more sensitive to gentamicin. I also examined how gentamicin affects the Arf1 pathway at the molecular level. My objective is to clarify the specific target of gentamicin in the intracellular trafficking pathway.

Aim 2 was to explore whether gentamicin can affect translation fidelity in eukaryotes, and lead to programmed ribosomal frameshifting efficiency changes *in vivo*. Studies have shown that gentamicin can suppress premature termination codons by disrupting translational fidelity (Sermet-Gaudelus, 2007). However, it has not been reported in the literature whether gentamicin can affect the efficiency of programmed

ribosomal frameshifting *in vivo*. Since *S. cerevisiae* is an ideal model system to study programmed ribosomal frameshifting (Harger & Dinman, 2003), my aim was to elucidate the programmed ribosomal frameshifting efficiency changes which may play a role in the translational regulation of gene expression.

Aim 3 was to identify all the gentamicin-binding proteins in *S. cerevisiae*. Some proteins have shown to potentially bind to gentamicin *in vitro* (Horibe et al., 2001; Miyazaki et al., 2004; Horibe et al., 2004). However, because of the poor resolution of one-dimensional SDS-PAGE, it still remains a question as to whether all the proteins that can bind to gentamicin have been identified. In our aim, with the power of mass spectrometry, we reasoned that lower abundant gentamicin-binding proteins would be identified.

Aim 4 was to test purified gentamicin congeners (including C_{1a}, C_1, C_2 and C_{2a}) on mutant strains with impaired Arf1 activity. Different congeners exhibit different cellular toxicity and nephrotoxicity *in vivo* (Sandoval et al., 2006). It is our interest to test the congeners in yeast *ARF1* mutant strains and Arf-GEF, or Arf-GAP mutant strains, and to clarify which congener is the least toxic. This result will help us to meet the long-term goal of developing practical therapeutic approaches for better using gentamicin.

MATERIALS AND METHODS

1 Media, Strains and Plasmids

1.1 Bacterial growth media and bacterial cell lysis

Bacterial strains (*E. coli*) were cultured at 37 ℃ in LB medium (1% Bactotryptone (*w/v*), 0.5% yeast extract (*w/v*), and 1% NaCl (*w/v*)) unless otherwise indicated. Ampicillin and other antibiotics were added after autoclaving as indicated. Solid LB medium was made with an additional final concentration of 2% agar (w/v).

Bacteria were lysed in 1 × PBS containing 1 × Complete protease inhibitor cocktail (Roche, Indianapolis, IN) lysis buffer unless otherwise indicated. 10 × PBS solution was made as a stock (1.4 *M* NaCl, 27 m*M* KCl, 101 m*M* Na_2HPO_4, and 18 m*M* KH_2PO_4, pH 7.3). To lyse bacterial cultures, freeze/thaw cycles were used with bacterial cell suspensions of up to 1.2 ml. Sonication was applied to bacterial cell suspensions with volumes larger than 1.2 ml.

For freeze/thaw cycle lysis, bacterial were collected by centrifugation (4,500 *g* for 10 minutes). Cells were resuspended in 1 × lysis buffer. 1 µl of 10 mg/ml lysozyme solution was added to each 100 µl of cell suspension, and mixed gently. Tubes were incubated at room temperature for 5 minutes. A liquid nitrogen bath and a warm water bath were prepared in separate ice buckets. Tubes containing lysozyme-treated cell suspensions were placed in liquid nitrogen bath until cells were frozen solid (~ 20 seconds). Then tubes were transferred to a flotation carrier and placed in a warm water bath until the suspension became fully liquid (~ 1 minute). This freeze/thaw cycle was repeated for 10 times. Benzonase® (Novagen) was added to a final concentration of 10 U/ml. Cell lysates with Benzonase® were incubated at room temperature for

10 minutes. Cell debris was removed by a high spin in a tabletop microcentrifuge for 10 minutes.

For sonication lysis, bacterial were collected by centrifugation (4,500 *g* for 10 minutes). Cells were resuspended in 1 × lysis buffer. 1 µl of 10 mg/ml lysozyme solution was added to each 100 µl of cell suspension, and mixed gently. Tubes were incubate**d** at room temperature for 5 minutes. Tubes were then transferred to ice and allowed to cool down for 30 minutes. Cells were sonicated on ice for 15 seconds followed by a 30 second interval. Sonication was repeated for 10 times. Benzonase® (Novagen) was added to a final concentration of 10 U/ml. Cell lysates with Benzonase® were incubated at room temperature for 10 minutes. Cell debris w**as** removed by a full speed spin in the tabletop microcentrifuge for 10 minutes.

1.2 Yeast growth media and yeast cell lysis

Yeast *S. cerevisiae* strains used in this study are listed in Table 1. Yeast cells were grown at 30 ℃ in YPD medium (1% yeast extract (*w/v*), 2% peptone (*w/v*), and 2% dextrose (*w/v*)) unless otherwise indicated. Solid YPD medium was made with an additional final concentration of 2% agar (*w/v*). Gentamicin was added to the medium to the indicated concentrations after autoclaving. The growth of each culture was determined by direct cell counting with the aid of a hemacytometer. Additional yeast growth media are presented as follows.

Synthetic defined (SD) medium contains 1.7% yeast nitrogen base (w/o $(NH_4)_2SO_4$) (*w/v*), 2% dextrose (*w/v*), and 0.5% $(NH_4)_2SO_4$ (*w/v*). Amino acid dropout mixtures were added after autoclaving. Solid SD medium was made with an additional final concentration of 2% agar (*w/v*).

Minimal synthetic medium contains 0.1% $(NH_4)_2SO_4$ (*w/v*), 0.05% $MgSO_4 \times 2H_2O$ (*w/v*), 0.087% KH_2PO_4 (*w/v*), 0.012% K_2HPO_4 (*w/v*), 0.01% NaCl (*w/v*), 0.01% $CaCl_2 \times 2H_2O$ (*w/v*), 0.83% succinic acid (*w/v*), 0.5% NaOH (*w/v*), 2% dextrose (*w/v*), 1% 100× vitamins (*v/v*), and 0.1% 1000× trace elements (*v/v*). To make the medium, NaOH was dissolve in water, which is about 80% of the desired volume. Succinic acid was added followed by all salts and water. Water was filled to 98.9% of the desired volume. After autoclaving and cooling of the medium to about 50 ℃, 100× vitamins (*v/v*) and 1000× trace elements (*v/v*) were added. 0.1% of complete amino acids mixture dropouts (*w/v*) can be added as the nitrogen source. Solid minimal synthetic medium was made with an additional final concentration of 2% agar (w/v).

Nitrogen-free minimal synthetic medium: 0.05% $MgSO_4$ (*w/v*), 0.087% KH_2PO_4 (*w/v*), 0.012% K_2HPO_4 (*w/v*), 0.01% NaCl (*w/v*), 0.01% $CaCl_2 \times 2H_2O$ (*w/v*), 0.83% succinic acid (*w/v*), 0.5% NaOH (*w/v*), 2% dextrose (*w/v*), 1% 100× vitamins (*v/v*), and 0.1% 1000× trace elements (*v/v*). To make the medium, NaOH was dissolve in water, which was about 80% of the desired volume. Succinic acid was added followed by all salts and water. Water was added to 98.9% of the desired volume. After autoclaving and cooling of the medium to about 50 ℃, 100× vitamins (*v/v*) and 1000× trace elements (*v/v*) were added. Repressing nitrogen sources including ammonia, glutamine, glutamate and asparagine were added. Derepressing nitrogen sources include urea, arginine, and proline can be added instead. Solid minimal synthetic medium was made with an additional final concentration of 2% agar (*w/v*).

The 100× vitamin solution (100 ml final) contains 0.4 m*g* Biotin, 0.2 m*g* folic acid, 400 m*g* inositol, 40 m*g* niacin, 80 m*g* pantothenic acid, 20 m*g* pABA, 80 m*g* pyridoxine-hydrochloride, 20 m*g* riboflavin, and 80 m*g* thiamine.

The 1000× trace elements solution (100 ml final) contained 100m*g* H_3BO_3, 100 m*g* KI, 100 m*g* $FeCl_3 \times 6H_2O$, 100 m*g* $CuCl_2 \times 2H_2O$, 100 m*g* $ZnCl_2$, and 100 m*g* $MnCl_2 \times 4H_2O$.

S. cerevisiae lysis buffer contains 0.15 *M* NaCl, 50 m*M* Tris-HCl (pH 7.5), 5 m*M* EDTA, 1% Triton X-100, and 1× Complete protease inhibitor cocktail (Roche, Indianapolis, IN) unless otherwise indicated. Mechanical disruption (glass beads beating) method was applied to lyse yeast cells. Yeast cells were collected by centrifugation at 4,500 *g* for 10 minutes. Cells were resuspended in 1× lysis buffer. Cells were resuspended in 200 µl fresh 1× lysis buffer and transferred to a 1.5 ml Eppendorf tube. Acid-washed glass beads (Sigma G-8772) were added to the meniscus level of lysis buffer. Tubes were cooled on ice for 10 minutes before being loaded onto the Mini-BeadBeater-8® (Biospec Products, Inc.). One minute of full speed rapid agitation was performed. Tubes were cooled on ice for 6 minutes. The beating/cooling cycle was repeated two more times. Cell debris was removed by centrifugation at 11,000 *g*, 4℃ for 40 minutes.

1.3 Plasmid DNA isolation from bacteria

Plasmid DNA was isolated from bacteria as follows by Qiagen Miniprep®. Briefly, a 5-10 ml bacteria overnight culture was spin down at 4,500 *g* for 10 minutes. Cells were resuspended in 1× lysis buffer and collected. Cells were resuspended in 250 µl Buffer P1 and transferred to a 1.5 ml Eppendorf tube. 250 µl of Buffer P2 was added

and mixed thoroughly by gently inverting the tube 4-6 times. 350 µl of Buffer N3 was added and mixed immediately and thoroughly by inverting the tube 4-6 times until the solution became cloudy. Cell debris was removed by centrifuging the tubes for 10 min at 11,000 *g*. The supernatant was applied to a QIAprep® spin column by pipetting. After centrifuging for 30-60 seconds, flow-through was discarded. The QIAprep® spin column was washed by adding 0.5 ml of Buffer PB and centrifuging for 30-60 seconds. Flow-through was discarded. QIAprep spin column was washed by adding 0.75 ml Buffer PE and centrifuging for 30-60 seconds. Flow-through was discarded, and the column was centrifuged for an additional minute to remove residual wash buffer. The QIAprep column was placed in a clean 1.5 ml microcentrifuge tube. To elute DNA, 50 µl of Buffer EB (10 m*M* Tris-HCl, pH 8.5) or water (for the purpose of DNA sequencing) was added to the center of each QIAprep spin column. The column was allowed to stand for one minute, and then centrifuged for one minute. Flow-through was collected and measured in Nanodrop® (Thermo Scientific) for the A_{260}/A_{280} and A_{260}/A_{230} ratios.

1.4 Plasmid DNA isolation from yeast

Plasmid DNA was isolated from yeast *S. cerevisiae* as described below. 5-10 ml of *S. cerevisiae* cells were cultured at 30 ℃ to reach mid-log phase. Cells were then harvested by centrifugation at 4,500 *g* for 5 minutes. Cells were resuspended in ice cold ddH_2O and respun. Cells were resuspended in 250 µl of Buffer P1 and transferred to a 1.5 ml Eppendorf tube. Acid-washed glass beads (Sigma G-8772) were added to the meniscus level of lysis buffer. The Eppendorf tube was cooled on ice for 10 minutes before sample was loaded onto the Mini-BeadBeater-8® (Biospec Products, Inc.). One minute of rapid agitation was performed. The tube was cooled on ice for 6 minutes. The

beating/cooling cycle was repeated 2 more times. Cell debris was removed by centrifugation for 40 minutes at 11,000 *g*, 4 ℃. The supernatant was transferred to a fresh 1.5 ml Eppendorf tube. 250 µl of lysis of Buffer P2 was added to the tube and mixed by inverting tube gently 4-6 times. The tube was incubated at room temperature for 5 minutes. 350 µl of neutralization Buffer N3 was added to the tube and mixed immediately but gently by inverting 4-6 times. After centrifuging the lysate for 10 minutes at maximum speed in a tabletop microcentrifuge, supernatant was transferred to a QIAprep® Spin Column by pipetting. The flow-through was discarded after centrifuging for 30-60 seconds. The QIAprep® Spin Column was washed by adding 0.75 ml of Buffer PE, followed by centrifugation for 30-60 seconds. Flow-through was discarded and column was centrifuged for an additional minute to remove residual wash buffer. The QIAprep Spin Column was placed in a clean 1.5 ml Eppendorf tube. To elute DNA, 25 µl of Buffer EB (10 mM Tris-HCl, pH 8.5) or water (for the purpose of DNA sequencing) was added to the center of each QIAprep® Spin Column. The column was allowed to stand for one minute, and was then centrifuged for one minute. The flow-through was collected and the O.D. was measured in a Nanodrop® (Thermo Scientific) for the A_{260}/A_{280} (1.8 for DNA, 2.0 for RNA) and A_{260}/A_{230} (should be very close to 2.0, otherwise consider contamination) ratio.

1.5 Bradford assay

Six standard solutions which contain 0, 1 µl, 2 µl, 4 µl, 8 µl or 10 µl of 1.4 mg/ml bovine serum albumin (BSA) were prepared. ddH_2O was added to each solution to a final volume of 800 µl. 200 µl of 1× Bradford Dye was added to each solution and mixed well. The absorbance at 595 nm was measured for each solution twice. A graph

plotting absorbance at 595 nm versus protein concentration was created. The graph was then used as a protein standard curve for further protein samples (Bradford, 1976).

The protein concentration of a sample was determined from its absorbance, using the standard curve to find the concentration of the standard which had the same absorbance as the sample. The protein concentration equation from the curve which was used in this study is: sample protein concentration = $A_{595nm} \div (n \times 0.068\ \mu g/\mu l)$ (n: n µl of samples were added to the 1 ml solution).

2 Transformations

2.1 Bacterial transformation

Bacteria cells are considered competent when the cells are able to take up DNA from the environment. To prepare competent bacteria cells, early log-phase growing bacteria were washed with $CaCl_2$, concentrated by centrifugation, and then resuspended in a small volume of $CaCl_2$. By doing this, the cell membrane of chemically competent bacteria is more permeable to DNA (Dagert & Ehrlich, 1979). The procedure is as follows.

A 100 µl aliquot of frozen *E. coli* DH5α cells was taken from -80 ℃ and inoculated into 50 ml of sterile LB broth. Antibiotics were not added since these cells do not have an antibiotic resistance-marker containing plasmid in them. Cells were grown at 37 ℃ with shaking until they reached an OD_{600nm} of 0.3 to 0.4 (1 cm pathlength). Cells were collected by centrifugation at 5,000 *g* for 10 minutes at 4 ℃. 100 m*M* $CaCl_2$ and 100 m*M* $MgCl_2$ solutions were cooled on ice. The bacteria cell pellet was gently resuspended on ice in 12.5 ml (1/4 of the initiated volume) of ice cold $MgCl_2$, taking 3 to 5 minutes for this procedure. Cells were collected by centrifugation at 4,000 *g* for 10

minutes. The bacteria cell pellet was resuspended on ice in 2.5 ml (1/20 of the initial volume) of ice cold $CaCl_2$, followed by an additional 22.5 ml (9/20 of the initial volume) of $CaCl_2$. The suspension was kept on ice for at least 20 minutes. Cells were collected by centrifugation at 4,000 *g* for 10 minutes and were resuspended in 1 ml (1/50 of the initial volume) of ice cold sterile 85 m*M* $CaCl_2$ in 15% glycerol (*w/v*). The competent cells were then ready for use.

Temperature changes affect competent cells greatly and reduce their efficiency over time. In the laboratory, we always prepare and store these cells in small aliquots to avoid free/thaw effects. It is recommended to dispense competent cells in 100 µl aliquots and freeze cells at -80 ℃. Always thaw and keep cells on ice prior to use (Chung, Niemela, & Miller, 1989).

DNA was added to an aliquot of competent cells, and then incubated on ice for 45 minutes. The cell-DNA mixture sample was taken (still in the ice bucket) to a 42 ℃ waterbath and heat shocked for 45 seconds. The temperature change in this heat-shock step should be very sudden, *i.e.*, from 0 ℃ directly to 42 ℃. The heat shock opens transient pores in the bacteria due to the destabilizing effect of the $CaCl_2$. The $CaCl_2$ in the transfection solution helps to bring the DNA close to the cell membrane. After 45 seconds at 42 ℃, the cells were plunged into an ice bath (from 42 ℃ directly to 0 ℃). It is important for the sample to go from 42 ℃ water back into an ice bath immediately after the heat shock. Cells were incubated on ice for 2 minutes to allow the transient pores in the bacteria to close, trapping the DNA inside the cells. 950 µl of LB medium was added to the transformation mixture without antibiotics. Cells were then cultured at 37 ℃ for 1 hour with shaking. During this time the antibiotic resistance genes are expressed, and

the number of colonies will be increased due to bacterial division. 10-100 μl of the culture was spread onto solid selectable media and incubated at 37 ℃ for 8 hours (Cohen, Chang, & Hsu, 1972) or overnight until colonies appear.

2.2 Yeast transformation

Yeast cells were cultured in 100 ml of YPD medium at 30 ℃ with shaking. At mid-log phase (8×10^6 cells/ml), the cells were harvested by centrifugation at 4,000 *g* for 5 minutes, washed once with TE buffer (pH 8.0), and resuspended in the same buffer to a final concentration of 2×10^8 cells/ml.

A 0.5 ml portion of the cell suspension was transferred to a test tube, and an equal volume of 0.2 *M* Li^+ (LiCl or Lithium acetate) was added. After one hour of incubation at 30 ℃ with shaking, 0.1 ml of the cell suspension was transferred to a 1.8 ml Eppendorf tube. 25 μl of plasmid DNA solution (670 μ*g*/ml) was added. The incubation continued for another 30 minutes at 30 ℃. An equal volume of sterilized 70% polyethylene glycol (PEG)-3250 was added and mixed thoroughly on a Vortex mixer. After standing for 1 hour at 30 ℃, the 1.7 ml Eppendorf tube was immersed into a 42 ℃ water bath and incubated for 5 minutes. The cells were cooled at room temperature, washed twice with room temperature ddH_2O, and resuspended again in 1.0 ml of ddH_2O.

The transformed cells were finally spread on selective media plates and incubated for at least 48 hours until the transformed strains formed colonies (Ito et al., 1983; Schiestl & Gietz, 1989).

3 SDS-PAGE and Western Blotting

3.1 SDS-PAGE

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis, SDS-PAGE, is a technique which is widely used in biochemistry to separate proteins according to their molecular mass. 7% SDS-PAGE gels were used to separate proteins from 75 kDa to 250 kDa, 10% SDS-PAGE gels were used to separate proteins from 30 kDa to 100 kDa, and 12% SDS-PAGE gels were used to separate proteins from 15 kDa to 70 kDa, respectively.

The 7% SDS-PAGE running gel was made with: 15.3 ml of H_2O, 7.5 ml of 1.5 *M* Tris-HCl (pH 8.8), 0.15 ml of 20% (*w/v*) SDS, 6.9 ml of acrylamide/bis-acrylamide (30%/0.8% *w/v*), 0.15 ml of 10% (*w/v*) ammonium persulfate (APS), and 0.02 ml of TEMED, which was added under a hood.

The 10% SDS running gel was made with: 12.3 ml of H_2O, 7.5 ml of 1.5 *M* Tris-HCl (pH 8.8), 0.15 ml of 20% (*w/v*) SDS, 9.9 ml of acrylamide/bis-acrylamide (30%/0.8% *w/v*), 0.15 ml of 10% (*w/v*) ammonium persulfate (APS), and 0.02 ml of TEMED, which was added under a hood.

The 12% SDS-PAGE running gel was made with: 10.2 ml of H_2O, 7.5 ml of 1.5 *M* Tris-HCl (pH 8.8), 0.15 ml of 20% (*w/v*) SDS, 12 ml of acrylamide/bis-acrylamide (30%/0.8% *w/v*), 0.15 ml of 10% (*w/v*) ammonium persulfate (APS), and 0.02 ml of TEMED, which was added under a hood.

The stacking gel was made with: 3.075 ml of H_2O, 1.25 ml of 0.5 *M* Tris-HCl (pH 6.8), 0.025 ml of 20% (*w/v*) SDS, 0.67 ml of acrylamide/bis-acrylamide (30%/0.8% *w/v*),

0.025 ml of 10% (*w/v*) ammonium persulfate (APS), and 0.005 ml of TEMED, which was added under a hood.

The cells were cultured to the desired cell density and lysed with mechanical lysis (glass bead beating). The amount of protein loaded in each lane was calculated based on the total protein concentration determined by Bradford assay. Normally, 12 µg of protein from a whole cell lysate or 2 µg of a pre-purified protein was loaded in each lane for a 1.5 mm Mini-Protein Gel (Biorad, CA). The loading buffer was added to each protein sample in a 1:4 ratio (*v/v*). The maximum loading capacity of the 1.5 mm thick Biorad Mini-Protein gels were 25 µl in each well. For the denatured SDS-PAGE samples, the protein and loading buffer mixture was heated at 80 ℃ for 5 minutes before being collected and loaded onto the SDS-PAGE gel.

SDS-PAGE gels were run at 60V through the stacking portion of the gel (about 20 minutes) and then at 110V through the resolving portion (about 1.5 to 2 hours) until the dye front reached the bottom of the gel.

3.2 Western Blotting

The SDS-PAGE gel was then transferred (wet transfer) to a nitrocellulose membrane at a constant voltage of 28V for 2 hours (room temperature) or 20V overnight (4 ℃). If the desired protein was of a large molecular weight (> 100 kDa), overnight transfer was always used.

The membrane was rinsed in KPBS-T buffer for 5 minutes before being blocked in KPBS-T buffer containing 5% non-fat dry milk for 1 hour at room temperature. The primary antibody was added to KPBS-T buffer containing 5% milk and the membrane

was incubated for 2 more hours at room temperature or overnight at 4 ℃. The primary antibody was diluted to 1:1000 or 1:2000 in this step.

The membrane was rinsed in KPBS-T buffer 3 times (10 minutes each time). Then the membrane was incubated with secondary antibody which had been diluted in 5% milk in KPBS-T buffer. The incubation lasted 1 hour at room temperature. The secondary antibody was already diluted to 1:5000 or 1:10000 in this step.

The membrane was washed in KPBS-T buffer 3 times (10 minutes each time). If the secondary antibody was HRP-conjugated, Amersham ECL detection reagent was applied to the membrane.

Finally, the membrane was exposed to the autoradiograph film for the desired amount of time (from 2 seconds to overnight).

3.3 Coomassie Blue Staining and Silver Staining

The Coomassie blue staining solution contained 2.5 g Coomassie brilliant blue R-250, 450 ml of methanol, 100 ml of glacial acetic acid, adjusted with water to 1 liter.

The Coomassie blue destaining solution contained 0.0075% (*v/v*) of glacial acetic acid and 10% (*v/v*) of ethanol in water.

Silver staining method was modified from the Witzmann laboratory's protocol (Lai et al., 2009). Only ddH_2O was used in making all solutions and wash buffers. Clean glass trays were used in every step. SDS-PAGE gels were fixed for at least 30 minutes in a solution of 50% methanol, 10% acetic acid (overnight is fine). SDS-PAGE gels were washed twice for 15 minutes each in a solution of 5% methanol and 1% acetic acid. SDS-PAGE gels were washed three times with water, 10 minutes each. SDS-PAGE gels were sensitized by treating with 0.2 g/L sodium thiosulfate solution for 90 seconds

followed by three 30 second rinses with water. SDS-PAGE gels were incubated in silver nitrate solution (2g/L) for 45 minutes. SDS-PAGE gels were rinsed three times in water, 20 seconds each. SDS-PAGE gels were developed using a solution with 60g/L of potassium carbonate, 0.05% (*v/v*) 37% formaldehyde, and 0.02g/L sodium thiosulfate. Developing usually takes ~ 5 minutes. The key is to stop developing process before background gets too dark. SDS-PAGE gels were stopped with 6% (*v/v*) acetic acid for 30 minutes. SDS-PAGE gels were rinsed with water before scanning.

4 Dual-luciferase Assay

4.1 Plasmids sequencing

Plasmids pYDL-control, pYDL-LA, and pYDL-TY1 were sequenced by automated dye-terminator sequencing. The primer designed to amplify the junction between the *ADH1* promoter and the *Renilla* luciferase gene was as follows: 5'-TTGTTCCAGAGCTGATGAGG. The primer designed to amplify the junction between the *Renilla* luciferase gene and the Firefly luciferase gene was as follows: 5'-CATGGCCTCGTGAAATCC. The primer designed to amplify the junction between the Firefly luciferase gene and the *CYC1* terminator was as follows: 5'-CCCGCTGAATTGGAATCG.

4.2 Luciferase Assay System®

Yeast stains harboring pYDL series of plasmids were inoculated into 5 ml of (-) uracil liquid medium at an OD595 = 0.7. Cells were harvested by centrifugation at 4,000 *g* for 10 minutes. Cells were resuspended in 1 ml of ice cold lysis buffer and washed once. The cells were resuspended in 0.3 ml of lysis buffer and lysed with the glass beads beating method. The Bradford assay was applied to determine protein

concentration. Luciferase activities were determined by using 5 µl of the lysate with the Luciferase Assay System® (Promega, Madison, WI).

The raw data readings were collected from a Monolight® 2010 Luminometer (Analytical Luminescence Laboratory). In each luminometer tube, 100 µl of LARII (from Dual-Luciferase® Reporter Assay System, Promega, Madison, WI) was predispensed. The luminometer was programmed. 5 µl of the cell lysate was transferred and mixed with LARII by pipetting/stirring. The firefly luciferase activity was measured in the luminometer. 100 µl of Stop and Glo Reagent was dispensed and mixed by pipetting/stirring. The *Renilla* luciferase activity was measured similarly (De Wet et al., 1985). Each sample was measured in triplicate.

5 GTP-bound *ARF1*-TAP Pull-down Assay

Plasmid pAB382 was kindly provided by Dr. P. Scott (University of Minnesota Medical School) (Scott et al., 2004). The gene encoding Gga2 (amino acid 1-326) was inserted into vector pGEX-5X-2 (Amersham Pharmacia, NJ) between the BamHI and XhoI sites. The plasmid encoded a fusion protein containing Gga2 in-frame with an amino terminal GST.

BL21 (DE3) bacterial cells transformed with pAB382 were cultured at 37 ℃ in 10 ml LB medium containing ampicillin (150 µg/ml) for 12-16 hours to reach confluence. This preculture was then diluted to 500 ml with fresh LB medium containing ampicillin (150 µg/ml) and also cultured at 37 ℃.

When the culture reached an OD_{600} of 0.6~0.8, isopropyl β-D-thiogalactoside (IPTG) was added to the culture medium to a final concentration of 0.8 m*M*, and the cells were incubated at 37 ℃ for an additional 5 hours. Cells were collected by centrifugation

(10 minutes at 4500 *g*, 4 ℃). Cell pellets were resuspended in lysis buffer and repelleted; then washed once and resuspended in 7 ml of ice-cold lysis buffer containing 20 m*M* Tris-HCl (pH 7.4), 100 m*M* NaCl, 0.1% (*w/v*) Triton X-100, and 1 × complete protease inhibitors (Roche, Indianapolis, IN). Cells were sonicated on ice 20 times for 15 seconds at 30 second intervals. The homogenate was then centrifuged (60 minutes at 15,000 *g*, 4 ℃) and the protein concentration of the supernatant was determined by Bradford assay.

Ten MicroSpin GST Purification Modules (Amersham Pharmacia, NJ) with 50 µl bed volume of Glutathione Sepharose 4B were prewashed with 300 µl of lysis buffer. 174 µg of protein from the supernatant was gently mixed with each MicroSpin column and incubated at 4 ℃ for 50 minutes to ensure optimal binding of GST-Gga2p$^{1\text{-}326}$ to the Glutathione Sepharose 4B matrix. After incubation, the flow-through of each MicroSpin column and four subsequent washes with 150 µl lysis buffer were discarded. 200 µg (adjusted to a final volume of 250 µl) of yeast *ARF1*-TAP strain whole cell lysate prepared from yeast cells grown to different cell densities and in the absence or presence of gentamicin were added to the MicroSpin columns and incubated at 4 ℃ for 30 minutes. The flow-through of each MicroSpin column and four consecutive washes with 150 µl lysis buffer were discarded. 40 µl of elution buffer (10 m*M* glutathione, 50 m*M* Tris-HCl pH 8.0) was added and final spin elutions were collected for SDS-PAGE and Western Blot analysis.

6 Spot Dilution Assay

S. cerevisiae strains were inoculated at 30 ℃ in liquid YPD medium unless otherwise indicated. Cell number was counted using a microscope and a hemacytometer at mid-log phase ($8 \times 10^6 \sim 2 \times 10^7$ cells/ml). Based on the cell counts, 4 µL of several cell

dilutions were placed side by side onto solid YPD media in the absence or presence of gentamicin. In brief, 4 μL of dilutions containing 10,000 cells, 1,000 cells, 100 cells, or 10 cells (or other cell numbers as indicated) were plated. Plate images were taken after an incubation at room temperature (or 30 ℃ as indicated) for approximately 2-3 days.

7 Carboxypeptidase Y Processing Assay

Wild-type or mutant *S. cerevisiae* strains were inoculated at their permissive temperature to early-log phase (5×10^6 cells/ml) before gentamicin (to a final concentration of 500 μg/ml) was added to the medium. After 0, 4 or 8 hours of additional incubation at their permissive temperature, aliquots of cells were removed, lysed and prepared for SDS-PAGE and Western Blot analysis using anti-CPY (Molecular Probes, Invitrogen) as the primary antibody.

8 Gentamicin-Sepharose 4B Affinity Binding Assay

CNBr-activated sepharose 4B (2 *g*; GE healthcare, NJ) was thoroughly swollen in 10 ml of 1m*M* HCl (to a final volume of 7 ml) and washed with 400 ml of 1 m*M* HCl in several aliquots followed by an additional wash with 20 ml of coupling buffer (0.1 *M* $NaCO_3$ pH 8.3, 0.5 *M* NaCl). The resin was separated into a control resin group and gentamicin-binding resin group. The control resin was mixed with 10 ml of the coupling buffer and incubated at 4 ℃ overnight with gentle rotation. The gentamicin-binding resin was mixed with 10 ml of gentamicin coupling buffer (10 m*M* gentamicin, 0.1 *M* $NaHCO_3$ pH 8.3, 0.5 *M* NaCl) and incubated under the same conditions as the control resin. For the gentamicin-binding resin, the excess gentamicin was removed by washing the resin with 40 ml of the coupling buffer. The remaining CNBr-activated groups on both resins were inactivated by incubation with blocking buffer (0.2 *M* glycine pH 8.0, 1% bovine

serum albumin) at 4 ℃ overnight with gentle agitation. The resins were then washed with three cycles of alternating pH (0.1 *M* acetic acid/sodium acetate (pH 4.0) containing 0.5 *M* NaCl and 0.1 *M* Tris-HCl (pH 8.0) containing 0.5 *M* NaCl). Each resin was equilibrated in PBS (0.05 *M* NaH_2PO_4, 0.05 *M* Na_2HPO_4, 0.2 *M* NaCl pH 7.4) before use.

400 μ*g* of yeast whole cell lysate (adjusted to a final volume of 2 ml) from mid-log phase yeast *ARF1*-TAP strain was added to 1 ml of either the control resin or the gentamicin-binding resin and incubated at 4 ℃ for 2 hours with gentle agitation. Each resin was washed four times with 10 ml of PBS buffer before a final elution with 6 ml elution buffer (0.05 *M* NaH_2PO_4, 0.05 *M* Na_2HPO_4, 1 *M* NaCl pH 7.4, 10 m*M* gentamicin) which was concentrated to 40 μl using a Vivaspin 500 column (MWCO 5kDa, GE Healthcare). The flow-through of the four washes and concentrated elution were collected for SDS-PAGE and Western Blotting analysis.

9 Mass Spectrometry Analysis of Gentamicin Binding Proteins Assay

A single colony of yeast strain BY4741 was inoculated overnight in liquid YPD at 30 ℃. The overnight culture was then diluted to 1×10^6 cells/ml by liquid YPD in the presence or absence of gentamicin (500 μg/ml). The subcultures were incubated overnight again at 30 ℃. Both subcultures were diluted to 2×10^6 cells/ml in liquid YPD in the presence or absence of gentamicin (500 μg/ml) on the next day, followed by an additional incubation of 5 hours until the subcultures reached mid-log growth phase with a cell density of 1×10^7 cells/ml. Cells were harvested by centrifugation and washed once with ice cold lysis buffer. Mechanical lysis (with glass beads beating) of the cells was performed. The supernatants of the cell lysates were collected for gentamicin affinity binding column and protein concentrations were determined by the Bradford assay.

A yeast lysate which contained 0.8 mg of protein was applied to either the gentamicin-affinity resin (1 ml) or the negative control resin (1 ml), and both resins were incubated at 4 ℃ overnight with gentle agitation. The columns were washed with 15 ml of washing buffer (0.2 *M* NaCl, 1.3 m*M* Ca^{2+}, 20 m*M* Tris-HCl pH7.6, 0.5 m*M* deoxycholate) before being eluted by 10 ml elution buffer (1 *M* NaCl, 10 m*M* gentamicin, 20 m*M* Tris-HCl pH 7.6, 0.5 m*M* deoxycholate). The elution was collected for mass spectrometric protein analysis.

The elution (10 ml) was concentrated by Vivaspin 500® 3 kDa MWCO (GE Healthcare) to a final volume of 500 µl. Proteins were reduced with dithiothreitol (DTT, to a final concentration of 10 m*M*) at 37 ℃ for 2 hours. After the samples were cooled to room temperature, iodoacetamide (to a final concentration of 50 m*M*) was added and samples were placed at room temperature for an additional 30 minutes in the dark. The samples were dialyzed against 10 m*M* ammonium bicarbonate using a Vivaspin 500® 3 kDa MWCO until the NaCl concentration was less than 0.1 m*M*. Trypsin was added at a 1:50 ratio (trypsin to protein ratio). Samples were digested overnight at 37 ℃.

The digested samples were analyzed by a Thermo-Finnigan linear ion-trap (LTQ) mass spectrometer coupled with a Surveyor autosampler (Thermo Scientific) and MS HPLC system. Each sample was injected twice onto a C18 microbore RP column (Zorbax SB-C18, 1.0 mm ×150 mm) at a flow rate of 50 µl/minute. The mobile phase A, mobile phase B, and mobile phase C were 0.1% formic acid in water, 50% acetone with 0.1% formic acid in water, and 80% acetone with 0.1% formic acid in water, respectively. The gradient elution profile was as follows: 10% B (90% A) for 5 minutes; 10-95% B (90-5% A) for 120 minutes; 100% C for 10 minutes; and 10% B (90% A) for

12 minutes. An initial parent mass scan of 400-2000 m/z was followed by 3 additional MS/MS scans of 200-2000 m/z with 35% of the normalized collision energy. Dynamic exclusion settings were as follows: the repeat count, 2; the repeat duration, 30 seconds; the exclusion duration, 120 seconds; the exclusion mass width, 0.75 m/z (low) and 2.0 m/z (high) (Lai et al., 2009).

The acquired data were searched against the modified yeast database created by M. Goebl from the SGD database using the SEQUEST (v. 28 rev. 12, Thermo Scientific) algorithms in Bioworks (v. 3.3, Thermo Scientific). General parameters were set as Lai and colleagues (Lai et al., 2009). The candidate peptides and proteins were validated by PeptideProphet (http://peptideprophet.sourceforge.net/) and ProteinProphet in the Trans-Proteomic pipeline (TPP, v. 3.3.0, Seattle Proteome Center, NHLBI Proteomics Center at the Institute for Systems Biology).

Biological replicates (n = 4) were applied for gentamicin-affinity binding resin and negative control resin.

RESULTS

CHAPTER 1: ARF1 PATHWAY IN THE GENTAMICIN RESPONSE OF SACCHAROMYCES CEREVISIAE

1 Gentamicin Sensitivity of *arf1^{ts} arf1*Δ *arf2*Δ Strains

The *arf1^{ts} arf1*Δ *arf2*Δ strains (gifts from Dr. Ken Sato, Molecular Membrane Biology Lab, RIKEN, Japan) used in this study are listed in Table 1. In this study, we examined whether the cells with reduced Arf1 activity were hypersensitive to gentamicin. Since *arf1*Δ *arf2*Δ double deletion cells are inviable, temperature sensitive (*ts*) alleles of *ARF1*, *arf1*-11, *arf1*-16 or *arf1*-18, were integrated at the *ADE2* locus in the chromosome (Yahara et al., 2001). The non-permissive temperature of *arf1*-11 *arf1*Δ *arf2*Δ and *arf1*-16 *arf1*Δ *arf2*Δ strains is over 30 ℃. The non-permissive temperature of *arf1*-18 *arf1*Δ *arf2*Δ strain is over 37 ℃ (Yahara et al., 2001).

The *arf1*-11 *arf1*Δ *arf2*Δ, *arf1*-16 *arf1*Δ *arf2*Δ and *arf1*-18 *arf1*Δ *arf2*Δ strains are representatives of three intragenic complementation groups (Yahara et al., 2001). The *arf1*-11 *arf1*Δ *arf2*Δ strain encodes an Arf1 with the following point mutations K38T, E132D, and L173S. None of these amino acid residues lies within a known Arf1 functional domain. E132D and K38T lie very close to two separated so-called Guanine Specific Regions, which can bind and stabilize guanine nucleotides. The phenotypes of the *arf1*-11 *arf1*Δ *arf2*Δ strain include very clear defects in ER-to-Golgi transport, which are due to the impaired recycling of ER-resident proteins from Golgi-to-ER retrograde trafficking (Yahara et al., 2001).

The *arf1*-16 *arf1*Δ *arf2*Δ strain encodes an Arf1 with the following point mutations D129E and E41V. In fact, D129 lies in a highly conserved domain

($N_{126}K_{127}X_{128}D_{129}$, where X indicates any amino acid), which is called the G-4 Region of all the small GTPases (Bourne et al., 1991). More specifically, eukaryotes have a conserved NKQD as their G-4 region in all the Arf proteins. The G-4 region plays a key role in the binding and stabilization of guanine nucleotides. The diploid strain that carries one allele of the triple deletion (K127, D129, E132) has a dominant lethal phenotype (Click et al., 2002). The other mutation site of the *arf1*-16 *arf1*Δ *arf2*Δ strain, E41, lies in the Switch1 Region (E_{41}VITTIPTIGFNVET), which is also recognized as one of the Sec7-binding domains and is highly conserved among all eukaryotic species (Mossessova et al., 1998). Actually, the Switch1 Region will undergo major conformational changes upon GTP binding and can bind to downstream effectors. For this reason, the Switch1 Region is also called the effector domain. The phenotypes of *arf1*-16 *arf1*Δ *arf2*Δ include defects in both Golgi-to-ER and ER-to-Golgi transport (Yahara et al., 2001).

The *arf1*-18 *arf1*Δ *arf2*Δ strain has an Arf1 point mutation at H80P. This point mutation lies in another Sec7-binding domain, GGQDRIRSLWRH_{80}, which is also recognized as the Switch2 Region (Mossessova et al., 1998). The intracellular transport remains normal in the *arf1*-18 *arf1*Δ *arf2*Δ strain. But cells from the *arf1*-18 *arf1*Δ *arf2*Δ strain have large stacks of the Golgi apparatus and numerous small vesicles inexplicably scattered throughout the cytoplasm for unknown reasons (Yahara et al., 2001).

As expected, our experiments found that at permissive temperature (room temperature), all of the $arf1^{ts}$ *arf1*Δ *arf2*Δ strains and the *ARF1 arf2*Δ strain had wild-type growth on YPD medium plate. However, at 37 ℃, all of the $arf1^{ts}$ *arf1*Δ *arf2*Δ strains exhibited temperature sensitive growth (Figure 4A) on YPD medium.

Spot dilution assays of the *ARF1 arf2*Δ and *arf1*^{ts} *arf1*Δ *arf2*Δ strains at permissive temperature (room temperature) are shown in Figure 4B. All strains grew well on YPD plates in the absence of gentamicin. For each strain, a serial dilution was applied to a YPD plate ranging from 1×10^7 cells/ml to 1.3×10^6 cells/ml. Although *ARF1 arf2*Δ cells were relatively unaffected by gentamicin, all of the *arf1*^{ts} *arf1*Δ *arf2*Δ cells were hypersensitive to gentamicin. The hypersensitivity generated by the three alleles was quite variable: *arf1*-11 *arf1*Δ *arf2*Δ and *arf1*-16 *arf1*Δ *arf2*Δ showed growth defects at a relatively low gentamicin concentration (100 μg/ml and 250 μg/ml); however, *arf1*-18 only showed mild growth defects at a much higher gentamicin concentration (500 μg/ml).

We also observed that single deletion strains of *arf1*Δ and *arf2*Δ were not sensitive to up to 500 μg/ml of gentamicin on the YPD medium plate.

From the literature, we knew that Arf1 and Arf2 are functionally interchangeable (Stearns et al., 1990). And in our experiments, we clearly show that in *arf2*Δ strains, the loss of Arf1 protein function can cause yeast cells to become hypersensitive to gentamicin.

These results suggest that *arf2*Δ cells with impaired Arf1 function are hypersensitive to gentamicin, and Arf1 is required for gentamicin resistance in *S. cerevisiae*. Next, we examined if gentamicin, by itself, can affect the intracellular protein expression level of Arf1, or affect the GTP-bound Arf1 to GDP-bound Arf1 ratio in yeast *S. cerevisiae*.

2 Change in GTP-bound Arf1 Protein Level during Different Cell Growth Phases

The Gga (γ-ear adaptin homology Golgi associated Arf-binding protein) family members are ubiquitous Golgi-localized clathrin-coated cargo adaptors (Puertollano et al., 2001). As mentioned previously, Gga2 protein binds exclusively to the GTP-bound form of Arf1 (Yoon et al., 2005).

In our experiments, we utilized yeast whole cell extracts to measure total Arf1 protein expression levels in different cell growth phases, and utilized a GST-labeled truncated yeast Gga family protein $Gga2^{1-326}$ to obtain a relative measurement of GTP-bound Arf1, also in different cell growth phases (Boman et al., 2002). The GST-$GGA2^{1-326}$ carrying plasmid pAB382 (Table 1) (kindly provided by Dr. P. Scott, University of Minnesota Medical School) (Scott et al., 2004) was transformed into bacteria BL21 (DE3) strain. After IPTG (0.3 *mM*) induction, GST-$Gga2^{1-326}$ protein was purified and later bound to GST-sepharose as described in Materials and Methods. Since the sepharose-bound GST-$Gga2^{1-326}$ only binds to GTP-bound Arf1, the Arf1 eluted from the sepharose by 10 *mM* glutathione is only the GTP-bound Arf1 in the whole cell extracts.

We used the yeast *S. cerevisiae* Arf1-TAP (*MAT***a** *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 ARF1::TAP-HIS3MX6*, Table 1) strain in this study because of the easy detection of Arf1 by Anti-TAP antibody (CAB1001, Open Biosystems) during Western Blotting. Cells of this Arf1-TAP strain were cultured in YPD medium or YPD + 500 μg/ml gentamicin medium to early-log growth phase (cell density 5×10^6 cells/ml), mid-log growth phase (cell density 1×10^7 cells/ml and 2.5×10^7 cells/ml), late-log growth phase (cell density 5×10^7 cells/ml) or stationary phase (1×10^8 cells/ml) before being harvested (Figure 5).

Firstly, we noticed that total Arf1 protein expression level remained constant, regardless of the absence or presence of gentamicin (500 μg/ml) in the media or the different growth phases of the cells (Figure 5B).

Secondly, the effect of gentamicin on GTP-bound Arf1 varied in the different growth phases (Figure 5A and 5C). When cells were in early-log growth phase and were to enter mid-log growth phase (cell density $5x10^6$ cells/ml and 1×10^7 cells/ml, Figure 5A), the relative abundance of GTP-bound Arf1 was dramatically reduced in cells inoculated with gentamicin (500 μg/ml). However, when cells were in mid-log growth phase and late-log growth phase (cell density $2.5x10^7$ and $5x10^7$ cells/ml, Figure 5A), there was no detectable difference in GTP-bound Arf1 levels in cells grown in the presence or absence of gentamicin. Finally, when cells were in stationary phase (cell density 1×10^8 cells/ml), gentamicin caused an increase in the relative abundance of the GTP-bound Arf1 (Figure 5A and 5C).

In conclusion, gentamicin (500 μg/ml) can affect the level of GTP-bound Arf1 in a cell growth phase-dependent manner, even though total Arf1 protein expression levels in yeast *S. cerevisiae* is not affected by gentamicin (500 μg/ml). At this point, it remains unclear whether any Arf1-GEF or Arf1-GAP function is affected by gentamicin. But our other studies suggest some possibilities which are presented later in this Chapter.

These results led us to investigate the gentamicin hypersensitivity of each gentamicin sensitive mutation strain in different growth phases.

3 Gentamicin Hypersensitivity of Gene Deletion Strains in Different Growth Phases

It has been reported that the loss of *SAC1*, *VPS52*, *VPS16*, *ZUO1*, or *VPS45* can result in gentamicin hypersensitivity in yeast *S. cerevisiae* (Wagner et al., 2006). In this study, we compared gentamicin sensitivity of all these single deletion strains, but in different growth phases, early-log growth phase, mid-log growth phase, and late-log growth phase. Cells of different strains from different growth phases were placed on YPD plates in the absence or presence of 50 or 100 μg/ml of gentamicin (Figure 6).

As a result, the *sac1*Δ strain showed gentamicin hypersensitivity on 50 μg/ml gentamicin plates. The growth phase affected gentamicin hypersensitivity only marginally. Compared with the previous liquid culture data, a 24-30 hours culture of the *sac1*Δ strain shows 80% growth inhibition in 50 μg/ml gentamicin + YPD medium (Wagner et al., 2006). The results suggest that *S. cerevisiae* cells are more sensitive to gentamicin in liquid broth.

The *vps52*Δ strain was sensitive to 50 μg/ml gentamicin and was more sensitive at mid-log phase or late-log phase on 100 μg/ml gentamicin plates (Figure 6). Compared with the previous liquid culture data, a 24-30 hour culture of the *vps52*Δ strain shows clear growth inhibition (over 90%) in 50 μg/ml gentamicin + YPD medium (Wagner et al., 2006).

The *vps16*Δ strain was sensitive to 100 μg/ml gentamicin. Cells were more sensitive at late-log growth phase and mid-log growth phase than early-log growth phase (Figure 6). At a lower gentamicin concentration (50 μg/ml), growth inhibition was not observed on plates (Figure 6). Compared with the previous liquid culture data, a 24-30

hour culture of the *vps1*6Δ strains showed growth inhibition (over 90%) in 50 μg/ml gentamicin + YPD medium (Wagner et al., 2006).

The *zuo1*Δ strain was sensitive to 50 μg/ml gentamicin and was more sensitive at late-log growth phase. Compared with the previous liquid culture data, a 24-30 hour culture of the *zuo1*Δ strain showed over 90% inhibition in 50 μg/ml gentamicin + YPD medium (Wagner et al., 2006).

The *vps45*Δ strain does not show growth inhibition in any growth phase in gentamicin as high as 500 μg/ml (data not shown). Compared with the previous liquid culture data, a 24-30 hour culture of the *vps45*Δ strain showed 50% growth inhibition in 50 μg/ml gentamicin + YPD medium, and a 90% growth inhibition in 500 μg/ml gentamicin + YPD medium (Wagner et al., 2006). But the growth inhibition was not detected on plates.

To summarize, cells from *sac1*Δ, *vps52*Δ, *vps16*Δ, and *zuo1*Δ strains have slightly different sensitivities to gentamicin in different cell growth phases (Figure 6). But overall gentamicin sensitivity effect is not highly related to the cells initial growth phases.

4 Impaired Arf1 Activity and Gentamicin Hypersensitivity

As mentioned previously, Arf1 is a small GTPase and GTP-bound Arf1 plays an essential role in the formation of transport vesicles. In fact, the switching between GTP-bound and the GDP-bound form Arf1 is tightly regulated by Arf-GEFs and Arf-GAPs. Here, we further investigate whether gentamicin has an effect on any Arf-GEFs or Arf-GAPs.

4.1 The loss of Arf-GEF function and gentamicin hypersensitivity

Sec7, Gea1 and Gea2, and Syt1 are known Arf1-GEF proteins in *S. cerevisiae*. The Sec7 domain is present in all Arf-GEFs in both *S. cerevisiae* and mammals. This domain is also the target of Brefeldin A, a fungal metabolite that can stabilize the abortive Arf1-GDP-Sec7 domain protein complex, thus preventing Arf1 from being activated through the Sec7 domain (Chardin & McCormick, 1999). In mammals, Arf-GEFs include the GBF1 family (mammalian orthologs of yeast Gea1 and Gea2), the BIG family (mammalian ortholog of yeast Sec7), the PSD family (mammalian ortholog of yeast Syt1), the IQSEC family (no yeast ortholog), the Cytohesin family (no yeast ortholog) and the FEXO8 family (no yeast ortholog) (Gillingham & Munro, 2007).

Among *SEC7*, *GEA1*, *GEA2*, and *SYT1*, *SYT*1 is not essential and the *syt*1Δ strain was not identified in the global screening of gentamicin sensitivity (Blackburn & Avery, 2003). Thus, we further investigated gentamicin sensitivity in *sec7*, *gea1*, and *gea2* mutants. As shown previously, while the wild-type strain was unaffected by gentamicin (Figure 7), a *zuo1*Δ strain was strongly inhibited.

SEC7 is an essential gene of *S. cerevisiae*. *SEC7* encodes a 226kDa Arf-GEF protein which includes a 23kDa Sec7 domain. Other than the Arf-GEF function, Sec7 is required for both mediating membrane tubule breakage and saccule fenestration at the *cis*-Golgi (Jackson, 2004) and the completion of ER-to-Golgi transport (Lupashin et al., 1996). For the latter, Sec7, with Ypt1, Sec19, Sly1, Uso1, and Sec18, are required for ER-derived transport vesicle to target the *cis*-Golgi membranes during the docking and tethering step which occurs before SNARE complex assembly and vesicle fusion

(Lupashin et al., 1996). The idea that Sec7 can direct and scaffold the binding and disassembly of the COPI-COPII switch has also been proposed (Deitz et al., 2000).

In our study, we utilized a strain containing the temperature-sensitive *sec7*-1 allele (gift from Dr. Peter Novick, Department of Cellular and Molecular Medicine, University of California, San Diego). The *sec7*-1 allele has mutations that lie outside of the Sec7 domain (Deitz et al., 2000) with a phenotype of blocked secretory pathway at the level of exit from the Golgi apparatus (Novick et al., 1980). Our studies show that the growth of the *sec7*-1 strain is not affected by gentamicin (Figure 7). This may suggest that the fission of tubules and fenestration of cisternal membranes are not significantly affected by gentamicin.

Yeast Gea1 and Gea2 are 50% identical and functionally redundant (Peyroche et al., 2001). Neither *GEA1* nor *GEA2* is an essential gene but the removal of both results in the loss of viability (Peyroche et al., 1996). Here in our study, we utilized temperature-sensitive alleles of *GEA1* (gifts from Dr. Jackson, Centre National de la Recherche Scientifique, France), *gea1-4 gea1*Δ*gea2*Δ and *gea1-6 gea1*Δ *gea2*Δ strains, to determine whether gentamicin affects cells with reduced Gea1 activity (Peyroche et al., 2001). Genomic *GEA1* and *GEA2* were knocked out in both mutation strains but a temperature sensitive *gea1* allele was integrated back into the genome in each strain (Table 1).

Both Gea1 and Gea2 are located in the *cis*-Golgi compartment and can recruit COPI-coated vesicles (Jackson, 2004). The *gea1-6 gea1*Δ *gea2*Δ and the *gea1-4 gea1*Δ *gea2*Δ mutants carry different mutations. The *gea1-4* allele carries multiple substitutions including two in the highly conserved Sec7 domain, while the *gea1-6* allele only carries

two amino acid substitutions downstream of the Sec7 domain (Peyroche et al., 2001). Both *ts* mutants have defects in the generation of COPI-coated vesicles from the Golgi and the *gea1-6 gea1*Δ *gea2*Δ mutant has a more severe phenotype with a dramatically slowed secretion of COPI-dependent proteins (Peyroche et al., 2001).

At their permissive temperature (32 ℃), the growth of the *gea1*^{ts} *gea1*Δ*gea2*Δ strains in the presence or absence of gentamicin are shown in Figure 7. Mid-log growth phase *gea1-6 gea1*Δ*gea2*Δ mutant show growth inhibition at 500 μg/ml gentamicin. Mid-log growth phase *gea1-4 gea1*Δ*gea2*Δ cells do not show detectable growth inhibition (Figure 7). Among the different growth phases, the growth of late-log growth phase *gea1-6 gea1*Δ*gea2*Δ cells is most affected by gentamicin (Figure 9); and *gea1-4 gea1*Δ *gea2*Δ cells do not show detectable growth inhibition by gentamicin in any growth phase (Figure 9).

In another study, it was proposed that even though Gea1 and Gea2 have overlapping functions, they are not redundant (Spang et al., 2001). One of the reasons was that a *gea1*Δ *arf1*Δ mutant is sicker than an *arf1*Δ strain but still viable, whereas *gea2*Δ *arf1*Δ cells are inviable (Spang et al., 2001). In the same paper, these investigators proposed that Sec7 could be the Arf-GEF solely responsible for Golgi-to-vacuole anterograde trafficking and *intra*-Golgi retrograde trafficking, and Gea2 is mainly responsible for the early Golgi-to-ER retrograde transport steps (Spang et al., 2001).

Despite the different views of the function of each Arf-GEF, here, we show that cells with impaired Arf-GEF function, especially impaired Gea1 and Gea2 function, is hypersensitive to gentamicin.

4.2 The loss of Arf-GAP function and gentamicin hypersensitivity

As mentioned previously, in *S. cerevisiae*, Gcs1, Glo3, Age1, and Age2 have been identified as Arf1-GAP proteins. In mammals, Arf-GAPs include the Arf-GAP1 family (mammalian ortholog of yeast Gcs1), the Arf-GAP3 family (mammalian ortholog of yeast Glo3), the SMAP family (mammalian ortholog of yeast Age2), and seven other Arf-GAP families without a yeast ortholog (Gillingham & Munro, 2007).

GCS1 was the first identified *S. cerevisiae* Arf-GAP encoding gene and Gcs1 has a cysteine-rich zinc finger, a so-called Arf-GAP domain (Cukierman et al., 1995; Poon et al, 1996). *GLO3*, *AGE1*, and *AGE2* were all identified later from a screening for high-copy suppressors of the loss of *ARF1* function mutants (Zhang et al, 1998). Sequence homology searches identified the same Arf-GAP domain in Glo3, Age1, and Age2 and all of the four proteins have shown Arf-GAP activity *in vitro* (Poon et al., 1996, 1999, 2001; Zhang et al., 2003). *SPS18* and *GTS1* encode proteins that also contain the cysteine-rich Arf-GAP domain but have no detectable Arf-GAP activity *in vitro* (Zhang et al, 1998).

The *gcs1*Δ mutants were found to be weakly sensitive to gentamicin (Blackburn & Avery, 2003). A number of studies have suggested that Gcs1 has a more critical role than the other Arf-GAPs (Poon et al., 1996, 1999, 2001; Zhang et al., 2003). Gcs1 expressing cells are still viable despite a triple deletion of *GLO3*, *AGE1* and *AGE2* (Zhang et al., 2003). Gcs1 is activated only when vesicles are close to fission, thereby ensuring that the inactivation of Arf1 is delayed sufficiently to allow the generation of vesicles (Antonny, 2006).

In *S. cerevisiae*, although the deletion of *AGE2* has no strong phenotype, cells lacking both *AGE2* and *GCS1* are inviable (Poon et al., 2001). The same study also suggested that Gcs1 and Age2 form an essential Arf-GAP pair (Poon et al., 2001) that provides an overlapping function for vesicle transport from the trans-Golgi network to the vacuole and late endosomes (Poon et al., 2001).

In a similar study from the same group, Gcs1 and Glo3 were proposed to form another Arf-GAP pair which provides an overlapping function for vesicle retrograde transport from the Golgi to the ER (Poon et al., 1999). Even though single deletions of *GCS1* or *GLO3* are viable, deletion of both *GLO3* and *GCS1* is lethal, which indicates that Glo3 and Gcs1 may at least partially substitute for each other (Poon et al., 1999). The single deletion of *GLO3* is viable but causes partial defects in Golgi-to-ER traffic (Poon et al., 1999).

We tested gentamicin sensitivity on the *gcs1*^{ts} *gcs1*Δ *age2*Δ mutant strain (PPY 164-5D, Table 1) and the *gcs1*^{ts} *gcs1*Δ *glo3*Δ mutant strain (PPY 147-28-2A, Table 1; both strains were kind gifts from Dr. Poon, Department of Biochemistry and Molecular Biology, Dalhousie University, Nova Scotia, Canada) at their permissive temperature (room temperature) (Figure 8). As we predicted, wild-type cells (BY4741, Table 1) are not affected by gentamicin. On plates with different concentrations of gentamicin, the *zuo1*Δ strain shows growth inhibition. The *gcs1-3 gcs1*Δ *age2*Δ strain was not affected by gentamicin at a concentration of up to 500 μg/ml. The *gcs1-28 gcs1*Δ *glo3*Δ strain shows clear growth inhibition at a gentamicin concentration of 250 μg/ml.

We also examineded the gentamicin sensitivity of *gcs1*^{ts} cells from different growth phases (Figure 9). The growth of *gcs1-28 gcs1*Δ *glo3*Δ cells from all growth phases (early-, mid-, and late-log growth phase) is affected by gentamicin (Figure 9); while *gcs1-3 gcs1*Δ *age2*Δ cells do not show detectable growth inhibition by gentamicin in any growth phase (Figure 9).

Age1 and Age2 are not functionally redundant since the double deletion of *AGE2* and *GCS1* is lethal (Poon et al., 2001) and growth of a strain with a double deletion of both *AGE1* and *AGE2* is indistinguishable from wild-type strains (Zhang et al., 2003). At this point, we only know that neither the *age1*Δ nor *age2*Δ strains were identified from the global screening for gentamicin sensitive strains (Blackburn & Avery, 2003).

This data supports the model that impaired Gcs1/Glo3 protein pair function leads to gentamicin hypersensitivity. Interestingly, this Arf-GAP pair has a partial block of vesicle retrograde transport from the Golgi to the ER.

5 Cellular CPY Processing in the Absence or Presence of Gentamicin

5.1 CPY processing in wild-type cells unaffected by gentamicin

Carboxypeptidase Y (CPY) is a soluble vacuolar hydrolase enzyme that removes amino acids from the carboxyl terminus of a protein or a peptide (Chiang & Schekman, 1991). On its way to the vacuole, CPY goes through several stages of post-translational modification and is sorted through the secretory pathway (Stevens et al., 1982). Since the 1980s, CPY has been a widely used reporter for monitoring the movement of proteins through the secretory pathway (Valls et al., 1987).

The processing pathway of CPY has been very well characterized (Losev et al., 2006; Valls, Hunter et al., 1987). CPY is synthesized as an inactive precursor protein and

translocated into the ER-lumen as a result of its 20-amino acid signal peptide. The signal peptide is then proteolytically removed and CPY undergoes dolichol-mediated core glycosylation in the ER-lumen. The addition of core oligosaccharides produces a 67 kDa premature ER form of CPY (pl form). During the transit of the p1 form of CPY through the Golgi complex, three of the four core oligosaccharides on CPY are elongated to produce carbohydrate side chains containing an average of 11 to 18 mannoses. The fully glycosylated premature Golgi form (p2 form) has a molecular mass of 69 kDa. Then CPY binds to its receptor, Vps10, and is sorted into vesicles at the late Golgi that are targeted to fuse with the prevacuolar/endosomal compartment. Upon arrival in the vacuole, the N-terminal propeptide segment of CPY is proteolytically removed to generate an active 61 kDa mature CPY (mature form). The recycling of CPY receptor, Vps10, is regulated by the retrograde trafficking pathway (Marcusson et al., 1994). The half-time for the maturation process has been shown to be approximately 6 minutes (Losev et al., 2006).

We examined whether cells with mutant forms of Arf1, Arf-GEFs, or Arf-GAPs have defects in CPY processing in the presence of gentamicin (Figure 10). Firstly, no premature CPY accumulation in the wild-type strain (BY47141) was seen, even after inoculation in gentamicin for up to 8 hours (Figure 10).

5.2 Disruption of cellular CPY processing in Arf1 or Arf1 regulator mutants by gentamicin

We do not observe premature CPY accumulation in the *arf1*Δ strain when cells were inoculated in YPD media (Figure 10), but, premature CPY accumulation of both p1 (ER) and p2 (Golgi) forms were observed in these cells after 4 hours of incubation in

YPD + gentamicin (500 μg/ml). After a longer incubation (8 hours) with gentamicin, an increased accumulation of both p1 (ER) and p2 (Golgi) premature forms of CPY was observed. For the *arf2*Δ strain, we do not observe premature CPY accumulation, even with up to 8 hours of incubation in gentamicin (500 μg/ml). This result is consistent with the literature that *ARF1* can complement the loss of *ARF2* but *ARF2* cannot complement the loss of *ARF1* since the *arf2*Δ strain displays no growth phenotype, but the *arf1*Δ cells grow slowly and are cold sensitive (Stearns, et al., 1990).

For the strains that lacked both *ARF1* and *ARF2* but were kept alive by the presence of a temperature sensitive allele of *ARF1* (*arf1*-11, *arf1*-16 or *arf1*-18, Table 1), we observed different premature CPY accumulation patterns before and during gentamicin incubation. We noticed very poor growth of both *arf1*-11 *arf1*Δ *arf2*Δ cells and *arf1*-16 *arf1*Δ *arf2*Δ cells after the addition of gentamicin. The *arf1*-11 *arf1*Δ *arf2*Δ strain had a very small amount of the p1 (ER) form of premature CPY already present before incubation with gentamicin. The accumulation of both p1 (ER) and an equal amount of p2 (Golgi) forms of premature CPY are observed after the addition of gentamicin (to a final concentration of 500 μg/ml). The *arf1*-16 *arf1*Δ *arf2*Δ strain had a similar amount of the p1 (ER) form of premature CPY before and during incubation in gentamicin (to a final concentration of 500 μg/ml). This phenomenon can be explained if the cells stopped growing soon after the addition of gentamicin. The *arf1*-18 *arf1*Δ *arf2*Δ strain did not have premature CPY accumulation when cells were incubated in YPD media. After 8 hours of incubation in gentamicin, premature CPY accumulation was observed for both p1 (ER) and p2 (Golgi) forms (Figure 10). These results also indicate that the *arf1*-11 *arf1*Δ *arf2*Δ and *arf1*-16 *arf1*Δ *arf2*Δ strains had ER to Golgi trafficking

blocked, even when cells were cultured in YPD medium. When gentamicin (500 μg/ml) was added to the YPD media, *arf1*-11 *arf1*Δ *arf2*Δ cells showed blocks to both ER to Golgi trafficking and inter/post-Golgi trafficking. The *arf1*-18 *arf1*Δ *arf2*Δ cells did not show blockage in intracellular trafficking when cultured in YPD medium, but cells showed blocks to both ER to Golgi trafficking and inter/post-Golgi trafficking after incubation in gentamicin (500 μg/ml) for 8 hours (Figure 10).

We also examined CPY processing in the Arf-GEF mutant strains. While in the single Arf-GEF deletion strains *SEC7*/*sec7*Δ (heterozygous) and *syt1*Δ, CPY processing was not blocked before or after incubation in gentamicin (to a final concentration of 500 μg/ml) (Figure 10), the *gea1*Δ strain accumulated premature CPY of both p1 (ER) and p2 (Golgi) forms but only after incubation in gentamicin (500 μg/ml). The result with the *gea1*Δ strain was also consistent with the *gea1*-4 *gea2*Δ and *gea1*-6 *gea2*Δ strains. Like the *SEC7*/*sec7*Δ strain, the *sec7*-1 strain did not show premature CPY accumulation, even with up to 8 hours of incubation in gentamicin (500 μg/ml) (Figure 10).

We then examined the CPY processing in the Arf-GAP mutant strains. While the *gcs1*Δ strain showed a similar amount of premature CPY accumulation in only the p1 (ER) form (before and after incubation in gentamicin), *glo3*Δ cells showed an increasing amount of premature CPY also in the p1 (ER) form only but after incubation in gentamicin (Figure 10). The *age2*Δ strain had premature CPY accumulation of both p1 (ER) and p2 (Golgi) forms only after incubation in gentamicin (500 μg/ml) (Figure 10). The CPY processing phenotype of *gcs1*-28 *gcs1*Δ *glo3*Δ cells was very similar to *gcs1*Δ cells and *glo3*Δ cells, and the CPY processing phenotype of *gcs1*-3 *gcs1*Δ *age2*Δ cells was very similar to *age2*Δ cells (Figure 10). These results indicated that the *gcs1*Δ,

*glo3*Δ and *gcs1*-28 *gcs1*Δ *glo3*Δ strains had ER to Golgi trafficking blocked before and during incubation in gentamicin (500 μg/ml). The *age2*Δ and *gcs1*-28 *gcs1*Δ *age2*Δ strains had blocks to both ER to Golgi trafficking and inter/post-Golgi trafficking when incubated with gentamicin (500 μg/ml) (Figure 10). Since Gcs1 and Glo3 work together as an Arf-GAP pair to provide an overlapping function for vesicle retrograde transport from the Golgi to the ER, and Gcs1 and Age2 work together as another Arf-GAP pair to regulate late Golgi-vacuole transport (Poon et al., 2001), the loss of *GLO3* or *AGE2* prevents specific trafficking steps which are regulated by these Arf-GAP pairs.

Arf1 is required for the budding of COPI-coated vesicles which requires coatomer, a stable cytosolic complex comprising seven equimolar subunits, α-, β-, β′-, γ-, δ-, ε-, and ζ-COP (Michelsen et al., 2007). Binding of Arf1-GTP to Golgi membranes leads to recruitment of the coatomer, deformation of the membrane, and budding of COPI-coated vesicles. COPI-coated vesicles mediate an essential and conserved retrieval pathway that continually recycles several classes of proteins, and lipids, from the *cis*-Golgi back to the ER (Eugster et al., 2004). It has been reported that the WD40 domains of the α-and β′-COPI subunits can recognize C-terminal di-lysine motifs (K(X)KXX) (where K is lysine and X is any amino acid) on ER membrane proteins (Eugster et al., 2004), and two highly conserved stretches in the β-and δ-COPI subunits can recognize Arginine (R)-based ER localization signals (Michelsen et al., 2007). When the ARF1 pathway is inhibited by gentamicin, COPI mediated retrograde trafficking is partially blocked. Thus, ER lumenal and ER membrane proteins fail to be retrieved from the Golgi back to the ER, which in return affects anterograde trafficking from the ER to the Golgi.

Thus, in some of the strains we tested which have defects in ARF1 pathway components, CPY trafficking and processing are further blocked by gentamicin (500 μg/ml) (Figure 17).

6 Enrichment of Arf1-TAP Protein by Gentamicin-binding Resin

One possible explanation for the effects of gentamicin on the ARF1 pathway is that gentamicin can directly bind to Arf1 or Arf1 effectors, thus affecting Arf1 activity *in vivo*. This hypothesis was inspired by the binding of Brefeldin A (BFA) to the Arf1-GDP-Sec7 complex (Chardin & McCormick, 1999). In order to test this hypothesis, yeast whole cell extract was incubated with gentamicin-bound resin or empty (negative control) resin.

Both resins were prepared as described in Materials and Methods. Briefly, after being incubated with the same yeast *ARF1*-TAP strain (Table 1) whole cell extract, both resins were washed four times with lysis buffer before a final elution with elution buffer (lysis buffer + 10 *mM* gentamicin).

The concept of gentamicin affinity-binding pull-down assays has been used in similar studies to identify the gentamicin binding proteins disulfide isomerase (Horibe et al., 2001), heat shock protein 90 kDa beta member (Horibe et al., 2004), calreticulin (Horibe et al., 2004) and heat shock 70 kDa protein (Miyazaki et al., 2004) in mammals. In this study, we modified the assay and applied yeast whole cell extract to the resin. We detected no protein bound to the empty (negative control) resin (details are included in Results: Chapter 3).

In our experiments, in different batches, GDP (to a final concentration of 10 m*M*) or GTPγS (to a final concentration of 10 m*M*) was premixed with yeast whole cell extract

before the incubation with resin. When GDP was added *in vitro*, the GDP concentration in the yeast whole cell extract was much higher (> 10^5 fold) than GTP, and the majority of Arf1 in the whole cell extract was in the GDP-bound form. With the *in vitro* addition of GTPγS (> 10^5 fold more concentrated than GDP in the whole cell extract), which is a nonhydrolyzable analog of GTP, the majority of Arf1 is in the GTP-bound form.

As shown in Figure 11, after three consecutive washes, no more non-specific bound Arf1-TAP protein was detected on the resins (Figure11A and 11B). And no Arf1-TAP protein was eluted from the empty (negative control) resin (Figure 11B). But Arf1-TAP was eluted from the gentamicin-bound resin by 10 m*M* gentamicin in the elution buffer (Figure 11A). The addition of GDP (10 m*M*) or GTPγS (10 m*M*) did not change the Arf1-TAP binding ability to the gentamicin-bound resin (Figure 11C and 11D).

We have attempted to use several yeast Arf1 antibodies unsuccessfully (Millipore, Anti-ARF1 monoclonal antibody, Cat # MAB3779; Chemicon, Anti-ARF1 monoclonal antibody, Lot # 25040787; Epitoics, ARF1 rabbit monoclonal antibody, Cat # 1635-1). We finally decided to test the binding ability of rat Arf1 to our gentamicin-bound resin and to detect possible rat Arf1 binding by our customized rabbit anti-ratArf1 antibody. Figure 11E shows convincingly that our rabbit anti-rat Arf1 antibody readily detects rat Arf1 (21KDa) while the rabbit pre-immune serum does not recognize rat Arf1 protein. We also noticed that after the incubation of rat cytosol with gentamicin-bound resin, there was no non-specific bound rat Arf1 protein remaining on the resin after the first of four consecutive washes (Figure 11F). But consistent with our previous yeast Arf1-TAP strain assay, rat Arf1 was eluted from the gentamicin-bound resin by elution buffer with

10 m*M* gentamicin. This result suggests that rat Arf1 is also capable of binding and is enriched by gentamicin-bound resin.

In brief, our results show that a gentamicin-bound resin can enrich for Arf1 *in vitro*. Furthermore, Arf1 binding to gentamicin-bound resin can be enhanced in a GTP/GDP-dependent manner. But from this experiment, we cannot tell whether Arf1 can bind to gentamicin directly or indirectly since the binding could be through an Arf1 effector from an Arf1 complex.

We have successfully expressed and purified N-terminal His-tagged yeast Arf1 protein (plasmid pLH-YARF1, Table 2) in *E. coli*, but the instrument $iTC_{200}^{®}$ (MicroCal, Piscataway, NJ) that we used to measure the direct binding of small molecules (gentamicin) to protein (N-terminal His-tagged yeast Arf1 protein) has not allowed us to detect a direct gentamicin-Arf1 interaction as of yet. We as yet do not know whether Arf1 protein can directly bind to gentamicin.

When the purified proteins (Arf1 or Sse1) were concentrated by VivaSpin 500, MWCO 3kDa (GE healthcare, NJ), the binding K_d of gentamicin to Sse1 or Arf1 was 23 μM and 77 μM, respectively. (For the measuring of Arf1 binding ability, [Arf1]=0.18mM and [gentamicin]=3.5mM; for the measuring of Sse1 binding ability, [Sse1]=0.055mM and [gentamicin]=1mM.) But since I noticed insoluble debris on the bottom of the VivaSpin tubes, I switched to VivaSpin 500, MWCO 10kDa (GE healthcare, NJ). No cell debris was observed any more, but I lost both binding titration curves as well. From Coomassie blue stained acrylamide gels, I did not see any difference by using Visaspin 500, MWCO 3kDa or Visaspin 500, MWCO 10kDa.

7 Gentamicin Sensitivity of *S. cerevisiae* Strains with only hArf4 or bArf1 as the Functional Arf

Arf protein function is conserved among eukaryotic organisms. In mammalian cells, there are six ARF proteins that have been identified. Even though *mARF1* and *yARF1* are only about 74% identical, each of the six mammalian *ARFs* can complement the deletion of both *yARF1* and *yARF2* (reviewed in Donaldson & Jackson, 2000). The six mammalian Arf proteins fall into three classes by sequence comparison (Kahn et al., 1991; Moss & Vaughan, 1995). Class I ARFs (Arf1-Arf3) are the best studied and appear to be functionally redundant. Class I ARFs control the formation of different vesicle coats in intracellular membrane transport. The function of class II ARFs (Arf4 and Arf5) is unclear. The only member of class III is Arf6 which is found on the plasma membrane and has been suggested to regulate membrane trafficking and cytoskeleton organization (Gillingham & Munro, 2007; Pucadyil & Schmid, 2009).

Studies showed that the expression of any human Arf, which includes hArf1, hArf2, hArf3, hArf4, hArf5, and hArf6, is able to rescue the lethality of yeast *S. cerevisiae arf1*Δ *arf2*Δ double mutants, which we studied in this experiment (Kahn et al., 1991).

Bovine Arf1 (100% identical to hArf1 in amino acid sequence) is a member of the class I ARFs and the hArf4 is a member of the class II ARFs (Gillingham & Munro, 2007; Pucadyil & Schmid, 2009). Strain 121.13C and strain RT166 (Table 1) were both kind gifts from Dr. Kahn (Kahn et al., 1991). In brief, in the strain 121.13C, b*ARF1* is expressed under a *GAL1* promoter from plasmid pJCB1-21, to make the yeast *arf1*Δ *arf2*Δ double deletion strain viable. In the strain RT166, h*ARF4* is expressed under the

same *GAL1* promoter from plasmid pJCH2-8, to make the yeast *arf1*Δ *arf2*Δ double deletion strain viable. Importantly, it has been shown that the level of h*ARF4* expressed under the control of the *GAL1* promoter in the absence of any yeast *ARF*'s is indistinguishable from that of y*ARF1* expressed from its own promoter (Kahn et al., 1991). The level of b*ARF1* expressed under the control of the same *GAL1* promoter in the absence of any yeast *ARF*s is 3-10 fold higher, but b*ARF1* product has a lower "specific activity" in yeast (Kahn et al., 1991).

We examined gentamicin sensitivity of the hArf4-expressing strain and the bArf1-expressing strain on YPGal plates in the presence or absence of gentamicin (Figure 12). The WT strain (BY4741), hArf4-expressing strain, and bArf1-expressing strains grow equally well on YPGal plates without gentamicin. However, while the growth of the WT strain is not affected by gentamicin (up to 500 μg/ml), bArf1-expressing strains are sensitive to gentamicin at concentrations of 250 μg/ml (and at even lower concentration as observed from liquid culture results). There was no significant sensitivity difference between cells from different growth phases.

From the phylogenetic analysis of the Arf cDNA sequences, yArf1 is more closely related to hArf1 (100% identical to bArf1 in amino acid sequence) than to hArf4 (Tsuchiy et al., 1991). And from amino acid sequence analysis, yArf1 is 77% identical to hArf1 and 72% identical to hArf4p, which are only slightly less than the identity between hArf1 and hArf4 (80%) (Welsh et al., 1994). Our results here show that Class I Arfs can be more sensitive to gentamicin than Class II Arfs.

8 Summary of the ARF1 pathway in Response to Gentamicin

A genetic screening by Blackburn and Avery revealed that at least 20 genes are required for gentamicin resistance in *S. cerevisiae* (Blackburn & Avery, 2003). Based on the genetic interactions between hypersensitive mutants and their products, we propose that the ARF1 pathway itself is a target of and is affected by gentamicin (Figure 3).

Firstly, since Arf1 and Arf2 are functionally interchangeable (Stearns et al., 1990) and we observed no gentamicin hypersensitivity in single deletion strains of either *arf1*Δ or *arf2*Δ, we applied spot dilution assays of *ARF1 arf2*Δ and $arf1^{ts}$ *arf1*Δ *arf2*Δ stains. The result showed that all of the $arf1^{ts}$ *arf1*Δ *arf2*Δ cells are hypersensitive to gentamicin (Figure 4) which indicates that the loss of Arf1 function can cause yeast cells to become hypersensitive to gentamicin.

Secondly, we noticed that while the total Arf1 expression level remained constant, regardless of the absence or presence of gentamicin (500 μg/ml) in the media or the different growth phases of the cells (Figure 5B), GTP-bound Arf1 varied with gentamicin (500 μg/ml) in the media in the different growth phases (Figure 5A and 5C). This result shows that gentamicin can affect the level of GTP-bound Arf1 in a cell growth phase-dependent manner, even though total Arf1 expression level in *S. cerevisiae* is not affected by gentamicin (500 μg/ml).

Thirdly, we showed that cells with impaired Arf-GEF or Arf-GAP functions are hypersensitive to gentamicin (Figure 7, Figure 8 and Figure 9). In particular, cells with mutations in the Gea1/2 proteins or the Gcs1/Glo3 protein pair are more sensitive to gentamicin (Figure 7, Figure 8 and Figure 9).

Fourthly, we examined whether cells with mutations in *ARF1*, *ARFGEF* genes, or *ARFGAP* genes had defects in intracellular traffic and CPY processing in the presence of gentamicin (Figure 10). We do not observe premature CPY accumulation in the wild-type strain (BY47141) after incubation in gentamicin for up to 8 hours, but in *arf1*Δ, *arf1-11 arf1*Δ *arf2*Δ, *arf1-18 arf1*Δ *arf2*Δ, *gea1*Δ, *gea1-6 gea2*Δ, *glo3*Δ and *age2*Δ strains, CPY trafficking and processing are further blocked by gentamicin (500 μg/ml) (Figure 10).

Fifthly, we showed that a gentamicin-bound resin can enrich both yeast Arf1-TAP protein and rat Arf1 protein *in vitro* (Figure 11). In yeast, Arf1 binding to gentamicin-bound resin is enhanced in a GTP/GDP-dependent manner (Figure 11C and 11D). But at present, we cannot tell whether Arf1 is binding to gentamicin directly.

Finally, we showed that in *S. cerevisiae* double deletion strains that lack both *ARF1* and *ARF2* but are kept alive by the presence hArf4 or bArf1 (100% identical to hArf1 in amino acid sequence), are hypersensitive to gentamicin (Figure 12). This result supports the idea that mammalian Arfs are more vulnerable to gentamicin and can at least partially explain the increased sensitivity of mammalian cells to gentamicin.

CHAPTER 2: INCREASE OF -1 PROGRAMMED RIBOSOMAL FRAMESHIFTING EFFICIENCY BY GENTAMICIN

1 Confirmation of the Dual-reporter Plasmid Sequence

Dr. Dinman (University of Maryland) has kindly provided us plasmids pYDL-control, pYDL-LA, and pYDL-TY1 (Table 2), for *in vivo* dual-luciferase assays (Harger & Dinman, 2003). Plasmids were amplified in the *E. coli* DH5α strain and confirmed by automated dye-terminator sequencing.

The sequencing results proved that the programmed ribosomal frameshifting signals utilized in the constructs were from yeast L-A virus (for the -1 Programmed Ribosomal Frameshifting signal, -1 PRF) (Fujimura et al., 1992) and Ty1 virus (for the +1 Programmed Ribosomal Frameshifting signal, +1 PRF) (Mellor et al., 1985). Plasmid sequencing results of dual-luciferase reporter genes are shown in Figure 2. The -1 PRF signal includes a special sequence, X XXY YYZ (the 0-frame is indicated by spaces), which is called a "slippery site". Also, a downstream secondary structure which usually forms an mRNA pseudoknot (as in pYDL-LA) is located immediately 3' to the slippery site. The pseudoknot can transiently cause the ribosome to pause during translation and the pause is required for the promotion of efficient -1 PRF (Figure 2B). Ty1 +1 PRF occurs when a peptidyl-$tRNA_{UAG}$ (Leu) slips +1 between CUU and UUA leucine codons (Figure 2C). This +1 PRF slippage occurs during a translational pause induced by slow recognition and low availability of the next in-frame peptidyl-$tRNA_{AGG}$ coding for arginine (Boeke et al., 1998).

2 Effect of Gentamicin on -1 Programmed Ribosomal Frameshifting

Yeast *S. cerevisiae* BY4741 strains harboring either pYDL-control (LL231) or pYDL-LA (LL232) were used in this experiment (Table 2). Cells were cultured in YPD or YPD + gentamicin (to a final concentration of 500 μg/ml) as indicated. Cells were harvested at mid-log growth phase. Raw data readings were collected with a Monolight® 2010 Luminometer (Analytical Luminescence Laboratory).

Encoded from the plasmid pYDL-control, the *Renilla* luciferase and Firefly luciferase are in the same reading frame and will always be translated into a fusion protein that has both *Renilla* luciferase and Firefly luciferase activity (Table 3 and Table 4). In contrast, encoded from the pYDL-LA, the *Renilla* luciferase and Firefly luciferase are not in the same reading frame. Without -1 PRF, the ribosome will meet a stop codon before reaching the coding sequence of Firefly luciferase. Thus, no functional Firefly luciferase will be expressed. In the case of a -1 PRF occurrence, *Renilla* luciferase and Firefly luciferase will be in the same reading frame again and will be translated into a fusion protein that has both *Renilla* luciferase and Firefly luciferase activity (Table 3 and Table 4).

The important measurement in this experiment is the ratio of Firefly luciferase to *Renilla* luciferase expression encoded from the L-A plasmid versus the same ratio encoded from the control plasmid, which represents the frequency of -1 PRF. Both ratios need to be normalized before further comparison (Table 5).

The experiments were carried out with a relatively large sample size (Table 3 and Table 4) to meet requirements for statistical analysis. In YPD, 14 replicates of the LL231 strain were tested (including one outlier that is not shown) and 31 replicates of the LL232

strain were tested (including three outliers that are not shown). In YPD + gentamicin (500 μg/ml) medium, 23 replicates of the LL231 strain were tested (including three outliers that are not shown) and 33 replicates of the LL232 strain were tested (including one outlier that is not shown).

The raw data were processed with Dr. Dinman's online version of the modified protocol "Systematic analysis of bicistronic reporter assay data" (Jacobs & Dinman, 2004). After the outliers were excluded from the raw data set, each set of data was tested and shown to be linear and normally distributed. The actual sample sizes were all above the "corrected sample size" (Kupper & Hafner, 1989). The statistical result showed that -1 PRF efficiency in the absence of gentamicin is 2.3 ± 0.1% (Table 5); while with 500 μg/ml gentamicin, the -1 PRF efficiency increases to 7.1 ± 0.2%, which is a 3-fold increase (Table 5). The -1 PRF efficiency of the no gentamicin set and the plus gentamicin set were statistically different, since the p-value was much less than 0.01 (8.17E-26), which corresponded to much less than a 1% chance of there being no statistical difference between the two sets.

3 Effect of Gentamicin on +1 Programmed Ribosomal Frameshifting

The *S. cerevisiae* BY4741 strains harboring either the pYDL-control (LL231) or the pYDL-TY1 (LL233) were used in this experiment (Table 2). Cells were cultured in YPD or YPD + gentamicin (to a final concentration of 500 μg/ml) as indicated. Cells were harvested in the mid-log growth phase.

The experimental design was the same as with the -1 PRF. The only difference was that the +1 PRF signal was inserted between the Firefly luciferase and the *Renilla* luciferase instead of the -1 PRF signal.

The experiments were also carried out with a relatively large sample size (Table 3 and Table 4). In YPD medium, 14 replicates of the LL233 strain were tested (including two outliers that are not shown). In YPD + gentamicin (500 μg/ml) medium, 26 replicates of the LL233 strain were tested (including one outlier that is not shown).

The raw data were processed in the same manner as the -1 PRF data. The statistical result shows that the +1 PRF efficiency in the absence of gentamicin is 11.0 ± 0.2% (Table 5); while with 500 μg/ml gentamicin, the +1 PRF efficiency decreases to 9.1 ± 0.2%, which is a 17% decrease (Table 5). The +1 PRF efficiencies of the no gentamicin set and the plus gentamicin set are statistically different, since the p-value is much less than 0.01 (2.01E-22), which corresponds to much less than a 1% chance of having no statistical difference between the two sets.

4 Documented Programmed Ribosomal Frameshifting of Yeast *S. cerevisiae*

Programmed ribosomal frameshifting was originally discovered in viruses and prokaryotes. But now, a growing number of examples have proved the existence of PRF signals in expressed eukaryotic genes (Lundblad & Morris, 1997; Morris & Geballe, 2000; Wills, Moore et al., 2006). In a pioneering study by Dr. Dinman's group, based on a computational sequence analysis, it was suggested that among the 6,353 Open Reading Frames (ORFs) of the yeast *S. cerevisiae*, 1,275 ORFs contained at least one strong -1 PRF signal (Jacobs et al., 2007). To prove the computational analysis, they tested selected candidates *in vivo* in the same study. The results indicated that eight out of nine -1 PRF signals tested promoted efficient -1 PRF *in vivo*.

The efficiency of programmed ribosomal frameshifting is affected by the host strain (either Wild-type or mutant strain) and the PRF signal that is being studied (Carr-

Schmid et al., 1999; Muldoon-Jacobs & Dinman, 2006). When using a wild-type *S. cerevisiae* strain as a host, in the study of L-A -1 PRF signals or Ty1 +1 PRF signals, 4% to 10% PRF efficiencies have been observed (Muldoon-Jacobs & Dinman, 2006; Jacobs et al., 2007).

Studies have also tested the PRF efficiency in mutant yeast strains, such as the *ssz1 zuo1* double deletion strain and the *ssb1 ssb2* double deletion strain (Muldoon-Jacobs & Dinman, 2006). The results showed the deletion of *ssz1 zuo1* or *ssb1 ssb2* inhibited -1 PRF but had no detectable effect on +1 PRF. The explanation for the inhibition of the -1 PRF was that impaired chaperone function causes nascent peptides to aggregate and blocks the polypeptide exit tunnel, and thus the movement of newly synthesized peptides into the tunnel. As a consequence, the peptidyl-tRNA 3' end is mis-positioned and both translation and frameshifting are partially inhibited (Muldoon-Jacobs & Dinman, 2006).

5 Consequences of Programmed Ribosomal Frameshifting Efficiency Changes

Notably, studies have pointed out that nearly all PRF events would directly lead translation elongation into premature termination, thus generating the hypothesis that PRF can be used to target mRNA for degradation via the nonsense-mediated mRNA decay pathway (NMD). In addition, cells could use *trans*-acting factors of PRF to post-transcriptionally regulate gene expression (Muldoon-Jacobs & Dinman, 2006). Considering the ORFs in the yeast *S. cerevisiae* that contain certain +1 PRF or -1 PRF signals, different PRF efficiencies may affect gene expression on a large scale.

CHAPTER 3: MASS SPECTROMETRY ANALYSIS OF GENTAMICIN BINDING PROTEINS

1 Comparison of Gentamicin-binding Proteins with Existing Data

From the eight gentamicin-bound resin elution injections (duplicate injections for each biological replicate), 67 proteins were reproducibly identified by LC-MS/MS (Table 6). These IDs were considered reproducible since proteins were identified by LC-MS/MS more than once. The identification of a protein is based upon the PeptideProphet and ProteinProphet algorithms. A protein was considered identified if the protein had 1) a protein probability value > 0.9; and 2) two or more unique peptides with each individual peptide probability value > 0.9. Proteins with only one unique peptide were excluded due to their higher false discovery rate.

Trypsin was the only protein that was identified from the eight empty (negative-control) resin elution injections (duplicate injections for each biological replicate). For mass spectrometry analysis, trypsin (porcine) is added to digest proteins into smaller peptides (Domon & Aebersold, 2006).

Previous studies have identified several gentamicin-binding proteins in mammals, including protein disulfide isomerase (PDI, UniProtKB: P05307) (Horibe et al., 2001), heat shock protein 90 kDa beta member (GRP94, UniProtKB: Q95M18) (Horibe et al., 2004), calreticulin (CRT, UniProtKB: P52193) (Horibe et al., 2004) and heat shock 70 kDa protein (HSP73, UniProtKB: CAN13333 and CAN87708) (Miyazaki et al., 2004).

In our study, many *S. cerevisiae* proteins with mammalian homologs were identified, such as Pdi1 (29% identity with PDI in amino acid sequence), Hsc82 and Hsp82 (46% identity with GRP94 in amino acid sequence) and Ssa1, Ssa2, Kar2, Ssb1,

Ssb2 and Sse1 (72%, 72%, 60%, 59%, 58% and 34% identity with Hsp73 in amino acid sequence, respectively), were all identified in our gentamicin-binding protein list (Table 6, column 5). Yeast protein Cne1 (the only yeast homologous of CRT, 30% identity in amino acid sequence) was not identified (Horibe et al., 2004), probably because in *S. cerevisiae*, Cne1 does not have Ca^{2+} binding ability (Xu et al., 2004).

2 Evaluation of the Gentamicin-binding Protein Dataset

Gentamicin-binding proteins were plotted against their protein expression levels (Figure 13A). Interestingly, the previously identified gentamicin-binding proteins all have relatively high expression levels (1E+05 to 1E+06 molecules/cell) (Horibe et al., 2004; Miyazaki et al., 2004). In our gentamicin-binding protein dataset, the protein expression levels distribute across a wider range, from 1E+03 molecules/cell to 1E+06 molecules/cell. Among the 55 proteins with expression data, 28 proteins were detected in more than three injections and the rest were detected in three or fewer injections. We noticed that some low-abundance proteins were detected more often than high-abundance proteins, which indicates protein detection is not only based on protein abundance.

Since smaller proteins with a relative molecular mass (*Mr*) of less than 35,000 Da are less likely to be identified by mass spectrometry (Krogan et al., 2006) (mainly because they have less peptides than bigger proteins), gentamicin-binding proteins were plotted against their molecular mass (Figure 13B). In our protein dataset, 43 proteins (over half of the proteins) were successfully identified by LC-MS/MS despite their low molecular masses (*Mr* < 35,000).

3 Identification of Functional Protein Complexes and Protein Networks

Proteins on our gentamicin-binding protein list could either directly or indirectly bind to the gentamicin molecule. To distinguish the gentamicin-binding proteins from their binding partners, we took advantage of a recently reported CYC2008 protein complex dataset (http://wodaklab.org/cyc2008/) (Pu et al., 2009). CYC2008 included 408 manually curated yeast complexes derived from small-scale studies in the current literature. Even though more protein complexes have been identified by large-scale high-throughput studies, this database fit our focus by organizing protein complexes according to their biological functions.

Based on the CYC2008 database, 33 gentamicin-binding proteins were grouped into four protein complexes: the eukaryotic elongation factor-1 (eEF-1) complex (Figure 14A), the ribosome-associated chaperone (RAC) complex (Figure 14C), the cytoplasmic ribosomal large subunit (Table 7) and the cytoplasmic ribosomal small subunit (Table 7). It is not surprising to discover that gentamicin has a strong interaction with ribosomal large and small subunits, maybe through rRNA. However, eEF-1 and RAC have never been reported to interact with gentamicin previously.

The Osprey software program (http://en.bio-soft.net/protein/Osprey.html) (Breitkreutz et al., 2003) was used to design an interaction network between eEF-1 and the remaining proteins that are on the gentamicin-binding protein list but had not been grouped into complexes yet (Figure 14A). The network revealed eight proteins, Yef3, Hyp2, Pab1, Sbp1, Mam33, Cmd1, Tpm1 and Ede1, that can be eEF-1 binding partners. In a systematic study of identification of protein complexes in *S. cerevisiae*, Abp1 and YKL056C encoding protein were found associated with Hyp2 and Ede1 in the same

protein complex (Costanzo et al., 2010). Bbc1 is in another protein complex with Hyp2 (Gruhler et al., 2002).

Both on the gentamicin-binding protein list, Hsc82 and Hsp82 are homologous to GRP94, which has shown binding ability with gentamicin in mammals in a previous study (Horibe et al., 2004). A protein-protein interaction network of Hsc82, Hsp82 and the remaining ungrouped proteins was developed (Figure 14B). Nine more proteins (Bmh1, Def1, Sba1, Vma4, Hsp60, Hcr1, Lat1, Sgt2 and Cdc37) were grouped as Hsc82 and Hsp82 binding partners.

RAC also showed interactions with other gentamicin-binding proteins via a network found using the Osprey software program (Figure 14C), especially the interactions between Ssa1, Ssa2, Ssb1 and Sse1, which all have homology to gentamicin-binding protein Hsp73 in mammals. This network may also explain the presence of Tsa1 on the gentamicin-binding protein list through an interaction with Sse1. A systematic study of the identification of protein complexes in *S. cerevisiae* also found that Clc1 can be in a protein complex with Sse1 (Gavin et al., 2002).

Finally, Pdi1, which is a homologue of the gentamicin-binding protein PDI, did not show an interaction with any of the remaining gentamicin-binding proteins.

By both functional protein complex analysis and protein-protein interaction networks, and as confirmation of many previous studies, 62 out of 67 proteins on our gentamicin-binding protein list were verified or grouped into protein complexes. The remaining ungrouped proteins are Mrp8, Rrs1, Crp1, Eno2 and Tif1. The evidence from this study is not enough to support whether they directly or indirectly bind to gentamicin.

Mrp8 is a protein with unknown function. Based on sequence analysis, Mrp8 is thought to be a mitochondrial ribosomal protein (Abraham et al., 1992).

Rrs1 binds specifically to the ribosome protein L11 (RPL11) (Miyoshi et al., 2004). Rpl11 was not on our gentamicin-binding protein list but it is in the cytoplasmic ribosomal large subunit complex with eighteen other proteins on our list.

Crp1 is a relatively unstudied protein. The only report about Crp1 stated Crp1 is a cruciform DNA (X-DNA) binding protein (Rass & Kemper, 2002).

Eno2 is a phosphopyruvate hydratase that catalyzes the conversion of 2-phosphoglycerate to phosphoenolpyruvate in glycolysis and the reverse reaction during gluconeogenesis (Gomes et al., 2008).

Tif1 (identical to Tif2) is a member of the translation initiation factor eIF4A (Neff & Sachs, 1999). Other members of the eIF4A complex, Cdc33 or Tif4631, were not identified.

CHAPTER 4: STUDIES OF FOUR GENTAMICIN CONGENERS

1 Effects of Gentamicin Congeners on Single Gene Deletion Strains

At the present time, clinically available gentamicin is a mixture of four major gentamicin congeners, C_1, C_{1a}, C_2 and C_{2a} (Figure 1). Studies have shown that different gentamicin congeners exhibit different cellular toxicities and nephrotoxicities *in vivo* (Sandoval et al., 2006), even though the congeners have very similar antimicrobial activities (Weinstein et al., 1967). However, separation of the gentamicin congeners is technically difficult and expensive. In this study, we started to examine the cellular toxicities of the congeners and tried to find the gentamicin congener least toxic to the mutants of our model system *S. cerevisiae*. The result of this study will help us to meet the long-term goal of developing practical therapeutic approaches for better using gentamicin in the clinic.

To begin the evaluation of how the gentamicin congeners affect *S. cerevisiae* , we selected four single gene deletion strains from *S. cerevisiae* that were previously found to be hypersensitive to gentamicin, *zuo1*Δ, *sac1*Δ, *vps52*Δ, and *vps16*Δ (Wagner et al., 2006). Plate spot dilution assays of each strain were performed on YPD, YPD containing 500 μg/ml gentamicin total mixture, or YPD containing 500 μg/ml gentamicin congener C_1, C_{1a}, C_2 or C_{2a} (Figure 15).

While the wild-type strain was unaffected by the gentamicin total mixture or any of the gentamicin congeners, mutant cells were all sensitive to the gentamicin total mixture and all gentamicin congeners. But clearly, the mutants were relatively more tolerant of gentamicin congener C_1 than the other gentamicin congeners and the

gentamicin total mixture (Figure 15). Interestingly, the gentamicin total mixture was more toxic than any single gentamicin congener.

At present, we do not have further data to explain why gentamicin C_1 is the least toxic gentamicin congener found in this study.

2 Effects of Gentamicin Congeners on Arf-GEF and Arf-GAP Mutant Strains

We also asked whether the different gentamicin congeners exhibit different levels of toxicity to yeast with impaired ARF1 pathway. From our earlier studies, strain *gea1-6 gea1*Δ *gea2*Δ and strain *gcs1-28 gcs1*Δ *glo3*Δ are hypersensitive to gentamicin (Results, Chapter 1). Here, we tested both of the strains on YPGal, YPGal containing 500 μg/ml gentamicin total mixture, or YPGal containing 500 μg/ml gentamicin C_1, C_{1a}, C_2 or C_{2a} (Figure 16). The results are consistent with the effects of the gentamicin congeners on single gene deletion strains, which is that mutant cells are less sensitive to gentamicin C_1 than the other gentamicin congeners or the gentamicin total mixture (Figure 16).

3 Effects of Gentamicin Congeners on *S. cerevisiae* Strains with only hArf4 or bArf1 as Functional Arf

Finally, we asked whether the different gentamicin congeners cause different toxicities to a yeast *arf1*Δ *arf2*Δ double deletion strain which is viable because of the expression of b*ARF1* or h*ARF4* under the *GAL1* promoter (Results, Chapter 1). Plate spot dilution assays were performed on both strains on YPGal, YPGal containing 500 μg/ml gentamicin total mixture, or YPGal containing 500 μg/ml gentamicin C_1, C_{1a}, C_2 or C_{2a} (Figure 16). These results are also consistent with the effects of the gentamicin congeners on single gene deletion strains and on the Arf-GEF or Arf-GAP mutant strains,

which is that mutant cells are less ssensitive to gentamicin C_1 than the other gentamicin congeners or the gentamicin total mixture (Figure 16).

So far, our conclusions regarding the toxicity of the four different gentamicin congeners is that gentamicin C_1 is the least toxic congener among C_1, C_{1a}, C_2 or C_{2a}, and the *vps52*Δ strain exhibited greater sensitivity to the gentamicin total mixture than any single congener (Figure 15).

CONCLUSIONS

The complete picture of how gentamicin induces nephrotoxicity involves multiple intracellular mechanisms. Gentamicin is taken up through the endocytic pathway and then trafficked in a retrograde manner through the Golgi to the ER, from where it is finally released to the cytosol (Kahn et al., 1991; Moss & Vaughan, 1995). It has been shown that gentamicin sequestered in the vacuoles or trafficked in a retrograde manner through the secretory pathway is nontoxic; however, the cytosol released gentamicin is toxic (Sandoval & Molitoris, 2004).

In our model system *S. cerevisiae*, inspection of the physical and genetic interactions of the gentamicin sensitive mutants revealed a network centered on the ARF1 pathway (Figure 17). Arf1 is a small GTPase and plays a key role in the regulation of retrograde and anterograde trafficking through the secretory pathway. Our studies show that *arf1*^{ts} *arf1*Δ *arf2*Δ cells, *gea1*^{ts} *gea1*Δ *gea2*Δ cells, and *gcs1*^{ts} *gcs1*Δ *glo3*Δ cells are all hypersensitive to gentamicin, which indicates that impaired Arf1 function causes yeast to become hypersensitive to gentamicin. As evidence, cellular CPY trafficking and processing are blocked by the presence of gentamicin in *arf2*Δ, *arf1-11 arf1*Δ *arf2*Δ, *arf1-18 arf1*Δ *arf2*Δ, *gea1*Δ, *gea1-6 gea1*Δ *gea2*Δ, *glo3*Δ, and *age2*Δ mutant strains. Interestingly, gentamicin can directly affect the level of GTP-bound Arf1 in a growth phase-dependent manner, even though the total Arf1 level is not affected. As predicted, we also find that gentamicin-bound resin can enrich both yeast Arf1-TAP protein and rat Arf1 protein *in vitro*. Furthermore, the binding of Arf1 to a gentamicin-bound resin is enhanced in a GTP/GDP-dependent manner. The gentamicin hypersensitivity is also observed in *S. cerevisiae* double deletion strains that lacked both *ARF1* and *ARF2* but are

kept viable by the presence of h*ARF4* or b*ARF1,* which supports the idea that some mammalian Arfs are more vulnerable to gentamicin and at least partially explains the increased sensitivity of mammalian cells to gentamicin.

It is also our interest to clarify the effect of gentamicin on programmed ribosomal frameshifting efficiency since it has not been reported in the literature. Our studies show that gentamicin increases -1 programmed ribosomal frameshifting efficiency by 3 fold but has virtually no effect on +1 programmed ribosomal frameshifting.

With the help of mass spectrometry, we also generated a gentamicin-binding protein list. Some of these proteins may contribute to gentamicin-induced intracellular toxicity.

Finally, a comparison of a gentamicin mixture and four of the gentamicin congeners revealed that gentamicin C_1 is less toxic than other gentamicin congeners or the gentamicin total mixture for single gene deletion strains, Arf-GEF and Arf-GAP mutant strains and strains with only hArf4 or bArf1 as functional Arf.

There are still questions that remain unclear after this study. Firstly, since Brefeldin A can stabilize the abortive Arf1-GDP-Sec7 domain complex and prevent Arf1 from being activated (Chardin & McCormick, 1999), we hypothesize that gentamicin has a similar effect on the Arf1-Gea1/Gea2 complex. This hypothesis may be also able to explain our observation that even though the total Arf1 level is not affected by gentamicin, GTP-bound Arf1 is significantly decreased at early-log and mid-log phase by the presence of gentamicin.

It has been known for decades that intensive reabsorption of proteins filtered by the renal glomeruli takes place in the renal proximal tubules (reviewed in Chambers,

2001). Also, the proximal tubule cell is one of the most endocytic mammalian cells. We also hypothesize that in general, in comparison to *S. cerevisiae*, human proximal tubule cells are more sensitive to gentamicin, and the proximal tubule cells with impaired Arf1 function are more vulnerable to gentamicin.

Table 1. Yeast strains used in this study.

Strain	Genotype	Reference
121.13C	*MATα ade2-101 his3-Δ200 leu2-3,112 lys2-801 ura3-52 arf1::HIS3 arf2::LEU2 (pJCB1-21*)Gal*$^{+}$	(Kahn et al., 1991)
APY022	*MATα ura3-52 leu2-3,112 his3-Δ200 lys2-801 ade2-101 gea1-6 gea2::HIS3*	(Peyroche et al., 2001)
BL2	*MATα* segregant of FY24XFY86	M. Goebl
BY1437	*MAT***a** *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 age2::KanMX4*	Open Biosystems
BY2783	*MAT***a** *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 vps16::KanMX4*	Open Biosystems
BY3835	*MAT***a** *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 arf2::KanMX4*	Open Biosystems
BY3890	*MAT***a** *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 arf1::KanMX4*	Open Biosystems
BY4318	*MAT***a** *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 vps52::KanMX4*	Open Biosystems
BY4462	*MAT***a** *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 vps45::KanMX4*	Open Biosystems
BY4741	*MAT***a** *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*	Open Biosystems
BY5062	*MAT***a** *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 sac1::KanMX4*	Open Biosystems
BY5512	*MAT***a** *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 syt1::KanMX4*	Open Biosystems
BY5937	*MAT***a** *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 zuo1::KanMX4*	Open Biosystems
BY6121	*MAT***a** *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 glo3::KanMX4*	Open Biosystems
BY6829	*MAT***a** *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 gea1::KanMX4*	Open Biosystems
BY23528	*MAT***a***/MATα his3Δ1/his3Δ1 leu2Δ0/leu2Δ0 ura3Δ0/ura3Δ0 met15Δ0/MET15 lys2Δ0/LYS2 sec7Δ::kanMX4/SEC7*	Open Biosystems
BY25401	*MAT***a***/MATα his3Δ1/his3Δ1 leu2Δ0/leu2Δ0 ura3Δ0/ura3Δ0 met15Δ0/MET15 lys2Δ0/LYS2 sec12Δ::kanMX4/SEC7*	Open Biosystems
BY7499684	*MAT***a** *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 ARF1::TAP-HIS3MX6*	Open Biosystems
CJY49-3-4	*MATα ura3-52 leu2-3,112 his3-Δ200 lys2-801 ade2-101*	(Peyroche et al., 2001)
CJY62-10-2	*MATα ura3-52 leu2-3,112 his3-Δ200 lys2-801 ade2-101 gea1-4 gea2::HIS3*	(Peyroche et al., 2001)
LLY231	*MAT***a** *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 pYDL-control*	This study
LLY232	*MAT***a** *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 pYDL-LA*	This study
LLY233	*MAT***a** *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 pYDL-TY1*	This study
NY966	*MATα sec7-1 ura3 leu2 his3*	P. Novick
NYY0-1	*MAT***a** *ade2 ::ARF1::ADE2 arf1::HIS3 arf2::HIS3 ura3 lys2 trp1 his3 leu2*	(Yahara et al., 2001)
NYY11-2	*MAT***a** *ade2 ::arf1-11::ADE2 arf1::HIS3 arf2::HIS3 ura3 lys2 trp1 his3 leu2*	(Yahara et al., 2001)
NYY16-1	*MAT***a** *ade2 ::arf1-16::ADE2 arf1::HIS3 arf2::HIS3 ura3 lys2 trp1 his3 leu2*	(Yahara et al., 2001)
NYY18-1	*MAT***a** *ade2 ::arf1-18::ADE2 arf1::HIS3 arf2::HIS3 ura3 lys2 trp1 his3 leu2*	(Yahara et al., 2001)
PPY164-5A	*MATα ura3-1 leu2-3,112 his3--11 ade2-1 trp1-1 can1-100 gcs1::URA3 pPP805-3*	(Poon et al., 1999)
PPY164-5B	*MATα ura3-1 leu2-3,112 his3--11 ade2-1 trp1-1 can1-100 age2::HIS3 pPP805-3*	(Poon et al., 1999)

PPY164-5C	*MATα ura3-1 leu2-3,112 his3--11 ade2-1 trp1-1 can1-100 pPP805-3*	(Poon et al., 1999)
PPY164-5D	*MATα ura3-1 leu2-3,112 his3--11 ade2-1 trp1-1 can1-100 gcs1::URA3 age2::HIS3 pPP805-3*	(Poon et al., 1999)
PPY147-28-2A	*MATα ura3-1 leu2-3,112 his3--11 ade2-1 trp1-1 gcs1::URA3 glo3::HIS3 pPP805-28*	(Poon et al., 1999)
PPY147-28-2C	*MATα ura3-1 leu2-3,112 his3--11 ade2-1 trp1-1 gcs1::URA3 pPP805-28*	(Poon et al., 1999)
PPY147-28-2D	*MATα ura3-1 leu2-3,112 his3--11 ade2-1 trp1-1 pPP805-28*	(Poon et al., 1999)
PPY147-28-8A	*MATα ura3-1 leu2-3,112 his3--11 ade2-1 trp1-1 glo3::HIS3 pPP805-28*	(Poon et al., 1999)
RT166	*MATα his3-Δ200 leu2-3,112 lys2-801 ura3-52 arf1::HIS3 arf2::LEU2 (pJCH2-8**)Gal*$^{+}$	(Kahn et al., 1991)

* b*ARF1* coding region behind the *GAL1* promoter.

** h*ARF4* coding region behind the *GAL1* promoter.

Table 2. Plasmids used in this study.

Plasmid Name	Vector	Yeast Marker	Bacterial Marker	Inserted Gene	Source
pAB382	pGEX5X	none	*amp*^{R}	*yGGA2*$^{1-326}$	(Boman et al., 2002)
pLH-hARF1	pET15B	none	*amp*^{R} *tet*^{R}	*hARF1*	This study
pLH-hARF4	pET15B	none	*amp*^{R} *tet*^{R}	*hARF4*	This study
pLH-yARF1	pET15B	none	*amp*^{R} *tet*^{R}	*yARF1*	This study
pLH-yENO2	pET15B	none	*amp*^{R} *tet*^{R}	*yENO2*	This study
pLH-yHSP82	pET15B	none	*amp*^{R} *tet*^{R}	*yHSP82*	This study
pLH-yHYP2	pET15B	none	*amp*^{R} *tet*^{R}	*yHYP2*	This study
pLH-ySSZ1	pET15B	none	*amp*^{R} *tet*^{R}	*ySSZ1*	This study
pLH-yTEF1	pET15B	none	*amp*^{R} *tet*^{R}	*yTEF1*	This study
pLH-yTIF1	pET15B	none	*amp*^{R} *tet*^{R}	*yTIF1*	This study
pLH-yZUO1	pET15B	none	*amp*^{R} *tet*^{R}	*yZUO1*	This study
pYDL-control	p416 ADH	URA3	*amp*^{R}	*Renilla* and Firefly luciferases (in the same reading frame)	(Harger et al., 2003)
pYDL-LA	p416 ADH	URA3	*amp*^{R}	*Renilla* and Firefly luciferases (in -1 reading frame)	(Harger et al., 2003)
pYDL-TY1	p416 ADH	URA3	*amp*^{R}	*Renilla* and Firefly luciferases (in +1 reading frame)	(Harger et al., 2003)

Table 3. Measurements of *Renilla* and Firefly luciferases intensity in YPD media.
S. cerevisiae strains LLY231 (pYDL-control), LLY232 (pYDL-LA) and LLY233 (pYDL-TY1) were used in this study (Table 1). For each sample, a single colony from one of the strains was picked up and cultured in 5 ml of YPD media overnight at 30 ℃. The overnight culture was diluted by YPD media to 2×10^6 cells/ml and incubated for another 6 hours at 30 ℃ to reach the mid-log phase (1×10^7 cells/ml). Cells were harvested and lysed. The cell lysate was then applied to the Dual-Luciferase Reporter® Assay System. The data are raw readings from the Monolight® 2010 Luminometer (Analytical Luminescence Laboratory).

Control Sample	Firefly RLU	Renilla RLU	F/R ratio
1	717796	2307771	0.311034
2	414405	1322295	0.313398
3	586402	1831500	0.320176
4	905938	2769500	0.327112
5	694758	2081138	0.333836
6	732090	2178084	0.336117
7	421932	1240683	0.34008
8	812577	2342299	0.346914
9	595495	1690852	0.352186
10	669172	1849428	0.361826
11	668397	1842390	0.362788
12	932510	2550584	0.365606
13	909530	2444292	0.372104
14	745376	1967815	0.378784

Ty1 Sample	Firefly RLU	Renilla RLU	F/R ratio
1	96170	2846411	0.033786
2	143285	4070607	0.035200
3	58084	1635092	0.035523
4	64985	1758197	0.036961
5	123929	3262937	0.037981
6	138644	3632607	0.038167
7	84243	2198075	0.038326
8	84243	2198075	0.038326
9	116317	3023119	0.038476
10	116249	2996642	0.038793
11	119066	3058200	0.038933
12	121291	3114520	0.038944
13	111189	2769590	0.040146
14	133793	3177878	0.042101

L-A Sample	Firefly RLU	Renilla RLU	F/R ratio
1	8022	1266186	0.006336
2	12388	1925884	0.006432
3	10503	1579967	0.006648
4	15329	2275735	0.006736
5	12894	1867712	0.006904
6	22026	3110498	0.007081
7	13833	1926832	0.007179
8	29570	3990270	0.007411
9	11956	1610156	0.007425
10	28261	3771724	0.007493
11	14201	1870128	0.007594
12	11215	1452647	0.00772
13	13005	1681021	0.007736
14	11220	1408544	0.007966
15	13555	1689092	0.008025
16	10812	1318009	0.008203
17	13683	1667882	0.008204
18	14232	1727467	0.008239
19	15403	1853376	0.008311
20	24893	2977446	0.008361
21	11713	1389255	0.008431
22	10790	1270140	0.008495
23	16416	1899885	0.008641
24	17895	2068124	0.008653
25	22166	2427183	0.009132
26	25455	2764349	0.009208
27	12653	1355571	0.009334
28	11962	1269407	0.009423
29	15795	1636081	0.009654
30	15555	1578738	0.009853
31	15222	1516713	0.010036

Table 4. Measurements of *Renilla* and Firefly luciferases intensity in YPD + 500 μg/ml gentamicin media. *S. cerevisiae* strains LLY231 (pYDL-control), LLY232 (pYDL-LA) and LLY233 (pYDL-TY1) were used in this study (Table 1). For each sample, a single colony from one of the strains was picked up and cultured in 5 ml of YPD (or YPD + 500 μg/ml gentamicin) media overnight at 30 ℃. The overnight culture was diluted by YPD (or YPD + 500 μg/ml gentamicin) media to 2×10^6 cells/ml and incubated for another 6 hours at 30 ℃ to reach the mid-log phase (1×10^7 cells/ml). Cells were harvested and lysed. The cell lysate was then applied to the Dual-Luciferase Reporter® Assay System. The data are raw readings from the Monolight® 2010 Luminometer (Analytical Luminescence Laboratory).

Control Sample	Firefly RLU	Renilla RLU	F/R ratio
1	630747	2449088	0.257544
2	804651	3082345	0.261052
3	487674	1815876	0.268561
4	927003	3422932	0.270821
5	795995	2934761	0.27123
6	713326	2626553	0.271583
7	605174	2205764	0.27436
8	2363307	8565024	0.275925
9	929589	3362538	0.276455
10	590974	2090421	0.282706
11	610200	2127193	0.286857
12	835458	2907143	0.287381
13	749989	2604095	0.288004
14	726489	2499858	0.290612
15	897658	3076759	0.291754
16	1185118	3972372	0.29834
17	576527	1925768	0.299375
18	618283	2045126	0.30232
19	694961	2296689	0.302593
20	878120	2884758	0.3044
21	452935	1415320	0.320023
22	2241837	6905755	0.324633
23	971055	2971475	0.326792

Ty1 Sample	Firefly RLU	Renilla RLU	F/R ratio
1	27411	1294608	0.021173
2	30062	1358901	0.022122
3	32410	1455926	0.022261
4	29301	1262561	0.023208
5	35355	1511759	0.023387
6	56122	2393392	0.023449
7	42216	1778025	0.023743
8	40027	1638626	0.024427
9	56195	2290520	0.024534
10	72749	2866700	0.025377
11	40800	1571500	0.025962
12	38330	1471835	0.026042
13	46589	1774488	0.026255
14	35972	1360378	0.026443
15	60968	2261809	0.026955
16	82847	3018492	0.027446
17	44632	1610610	0.027711
18	54652	1945162	0.028096
19	40215	1418169	0.028357
20	37855	1293899	0.029257
21	59430	2030684	0.029266
22	43395	1458535	0.029752
23	48025	1607887	0.029868
24	41310	1376125	0.030019
25	62706	2079921	0.030148
26	60128	1967387	0.030562

L-A Sample	Firefly RLU	Renilla RLU	F/R ratio
1	3051	194881	0.015656
2	6054	374748	0.016155
3	2370	143394	0.016528
4	62434	3592685	0.017378
5	3277	186388	0.017582
6	35256	1972481	0.017874
7	5965	331178	0.018011
8	6696	368393	0.018176
9	4585	252021	0.018193
10	58912	3227948	0.018251
11	2969	156404	0.018983
12	6256	326406	0.019166
13	6151	314010	0.019589
14	70212	3578533	0.019620
15	5182	262910	0.019710
16	70394	3564246	0.019750
17	51632	2576480	0.020040
18	5527	275058	0.020094
19	7401	355240	0.020834
20	75418	3522338	0.021411
21	4832	222223	0.021744
22	5672	259004	0.021899
23	5863	264379	0.022176
24	5876	262352	0.022397
25	59505	2655739	0.022406
26	7082	311658	0.022724
27	5787	253072	0.022867
28	75390	3274909	0.02302
29	73423	3166655	0.023186
30	90762	3880058	0.023392
31	139469	5868686	0.023765
32	6112	246061	0.024839
33	6681	260280	0.025669

Table 5. Systematic analysis of the -1 PRF and +1 PRF efficiency in *S. cerevisiae* in the presence or absence of gentamicin (500 μg/ml). The raw data of *Renilla* and Firefly luciferases intensity measurements are presented in Table 3 and Table 4. Gentamicin caused a significant increase in -1 PRF (200%) but virtually no effect on +1 PRF.

	Without Gentamicin			With Gentamicin		
	Control	+1 PRF	-1 PRF	Control	+1 PRF	-1 PRF
Firefly/*Renilla* luciferase intensity ratio	0.344	0.038	0.008	0.289	0.026	0.020
Frameshifting Efficiency		11.0 ±0.2%	2.3 ±0.1%		9.1 ±0.2%	7.1 ±0.2%

Comparing gentamicin treated and untreated cells	+1 PRF	-1 PRF
Percentage change	-17%	+200%
p-value	2.01×10^{-22}	8.17×10^{-26}

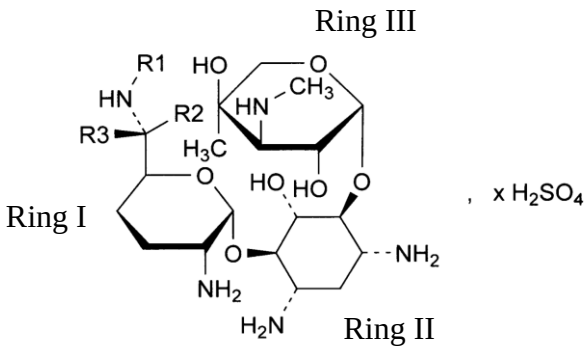
Table 6. Summary of considered gentamicin-binding proteins from LC-MS/MS. A total of 67 proteins were considered identified by LC-MS/MS as gentamicin-binding proteins. All 67 proteins are listed here by their systematic names. Data of protein subcellular localization, abundance and size are from the *Saccharomyces* genome Database (http://www.yeastgenome.org/).

Protein No.	ORF	Standard Name	Subcellular localization	Identified Before	Protein Molecules/cell	Protein Size
1	YBL072C,YER102W	RPS8	Cytoplasm		2E+04	22 kDa
2	YBR031W,YDR012W	RPL4	Cytoplasm		2E+05	39 kDa
3	YCR088W	ABP1	Punctate/Actin		6E+02	66 kDa
4	YDL082W,YMR142C	RPL13	Cytoplasm		1E+05	23 kDa
5	YDL191W,YDL136W	RPL35	Cytoplasm		2E+04	14 kDa
6	YDL229W,YNL209W	SSB1,SSB2	Cytoplasm	√	2E+05	67 kDa
7	YDR382W	RPP2B	Cytoplasm		6E+05	11 kDa
8	YEL054C,YDR418	RPL12	Cytoplasm		7E+04	18 kDa
9	YER074W,YIL069C	RPS24	Cytoplasm		6E+03	15 kDa
10	YGR167W	CLC1	Punctate/Late Golgi		3E+03	27 kDa
11	YHL034C	SBP1	Cytoplasm		1E+04	33 kDa
12	YHR064C	SSZ1	Cytoplasm	√	7E+04	51 kDa
13	YHR146W	CRP1	Cell periphery		3E+03	51 kDa
14	YKL056C	YKL056C	Cytoplasm		3E+04	19 kDa
15	YKL117W	SBA1	Cytoplasm/Nucleus		3E+04	24 kDa
16	YKL180W,YJL177W	RPL17	Cytoplasm		2E+04	21 kDa
17	YKR059W,YJL138C	TIF1,TIF2	Cytoplasm		1E+05	45 kDa
18	YLL024C	SSA2	Cytoplasm/Nucleus	√	4E+05	69 kDa
19	YML024W,YDR447C	RPS17	Cytoplasm		3E+04	16 kDa
20	YML028W	TSA1	Cytoplasm/Punctate		4E+05	22 kDa
21	YMR186W,YPL240C	HSC82,HSP82	Cytoplasm	√	4E+05	81 kDa
22	YNL079C	TPM1	Unlocalized		3E+03	24 kDa
23	YOL039W	RPP2A	Cytoplasm		4E+05	11 kDa
24	YOL121C,YNL302C	RPS19	Cytoplasm		3E+04	16 kDa
25	YOR007C	SGT2	Cytoplasm		9E+03	37 kDa
26	YPL090C,YBR181C	RPS6	Cytoplasm		7E+04	27 kDa
27	YPL106C	SSE1	Cytoplasm	√	7E+04	77 kDa
28	YPR080W,YBR118W	TEF1,TEF2	Cytoplasm		4E+02	50 kDa
29	YAL005C	SSA1	Cytoplasm	√	3E+05	70 kDa
30	YBL047C	EDE1	Punctate		1E+03	151 kDa
31	YDL075W,YLR406C	RPL31	Unlocalized		6E+04	13 kDa
32	YDR099W,YER177W	BMH2,BMH1	Cytoplasm/Nucleus		2E+05	31 kDa
33	YDR168W	CDC37	Cytoplasm		1E+04	58 kDa
34	YER056C-A,YIL052C	RPL34	Cytoplasm		2E+04	14 kDa
35	YER165W	PAB1	Cytoplasm		2E+05	64 kDa
36	YGL031C,YGR148C	RPL24A,RPL24B	Cytoplasm		2E+05	18 kDa

37	**YGR285C**	**ZUO1**	**Cytoplasm**		**9E+04**	**49 kDa**
38	YHL033C,YLL045C	RPL8A,RPL8B	Cytoplasm		2E+05	28 kDa
39	YHR174W	ENO2	Cytoplasm		3E+03	47 kDa
40	YHR203C	RPS4	Cytoplasm		1E+05	29 kDa
41	YIL070C	MAM33	Mitochondrion		5E+03	30 kDa
42	YJL034W	KAR2	Ambiguous	√	3E+05	74 kDa
43	YKL054C	DEF1	Cytoplasm		3E+03	84 kDa
44	YKL142W	MRP8	Cytoplasm		2E+03	25 kDa
45	YLR048W	RPS0B	Cytoplasm		6E+04	28 kDa
46	YLR061W	RPL22A	Cytoplasm		6E+04	14 kDa
47	YLR192C	HCR1	Cytoplasm		2E+04	30 kDa
48	YLR249W	YEF3	Cytoplasm		9E+05	116 kDa
49	YMR194W,YPL249C-A	RPL36	Cytoplasm		5E+03	11 kDa
50	YNL071W	LAT1	Mitochondrion		5E+03	52 kDa
51	YNL178W	RPS3	Unlocalized		1E+05	27 kDa
52	YOR096W	RPS7A	Cytoplasm		4E+04	22 kDa
53	YOR332W	VMA4	Vacuolar membrane		2E+04	26 kDa
54	YPL081W,YBR189W	RPS9	Cytoplasm		1E+05	22 kDa
55	YPL143W,YOR234C	RPL33	Unlocalized		7E+04	12 kDa
56	YAL003W	EFB1	Unlocalized		Not visualized	23 kDa
57	YBR109C	CMD1	Bud neck/cell periph./bud		Not visualized	16 kDa
58	YCL043C	PDI1	Cytoplasm/vacuole	√	Low signal	58 kDa
59	YEL034W	HYP2	Unlocalized		Not visualized	17 kDa
60	YGL103W	RPL28	Unlocalized		Not visualized	17 kDa
61	YJL020C	BBC1	Unlocalized		Not visualized	128 kDa
62	YJR123W	RPS5	Unlocalized		Not visualized	25 kDa
63	YLR259C	HSP60	Mitochondrion		Not visualized	61 kDa
64	YNL069C	RPL16B	Unlocalized		Not visualized	22 kDa
65	YOL127W	RPL25	Bud neck/ cytoplasm/cell periph.		Not visualized	16 kDa
66	YOR294W	RRS1	Unlocalized		Not visualized	23 kDa
67	YOR369C	RPS12	Unlocalized		Not visualized	16 kDa

Table 7. Gentamicin-binding proteins of the cytoplasmic ribosomal large subunit and the cytoplasmic ribosomal small subunit.

Cytoplasmic ribosomal large subunit		Cytoplasmic ribosomal small subunit	
YEL054C,YDR418	RPL12	**YLR048W**	RPS0B
YDL082W,YMR142C	RPL13	**YOR369C**	RPS12
YNL069C	RPL16B	**YML024W,YDR447C**	RPS17
YKL180W,YJL177W	RPL17	**YOL121C,YNL302C**	RPS19
YLR061W	RPL22A	**YER074W,YIL069C**	RPS24
YGL031C,YGR148C	RPL24A,RPL24B	**YNL178W**	RPS3
YOL127W	RPL25	**YHR203C**	RPS4
YGL103W	RPL28	**YJR123W**	RPS5
YDL075W,YLR406C	RPL31	**YPL090C,YBR181C**	RPS6
YPL143W,YOR234C	RPL33	**YOR096W**	RPS7A
YER056C-A,YIL052C	RPL34	**YBL072C,YER102W**	RPS8
YDL191W,YDL136W	RPL35	**YPL081W,YBR189W**	RPS9
YMR194W,YPL249C-A	RPL36		
YBR031W,YDR012W	RPL4		
YHL033C,YLL045C	RPL8A,RPL8B		
YOL039W	RPP2A		
YDR382W	RPP2B		



Gentamicin	Mol. Formula	R1	R2	R3
C1	$C_{21}H_{43}N_5O_7$	CH_3	CH_3	H
C1a	$C_{19}H_{39}N_5O_7$	H	H	H
C2	$C_{20}H_{41}N_5O_7$	H	CH_3	H
C2a	$C_{20}H_{41}N_5O_7$	H	H	CH_3
C2b	$C_{20}H_{41}N_5O_7$	CH_3	H	H

Figure 1. Chemical structures of gentamicin sulfate salt C_1, C_1a, C_2, C_2a and C_2b. The amine groups of gentamicin sugar rings exhibit variable p*Ka* values that range from 5.6 to 9.5. The amine groups on ring I are the most basic (p*Ka* ~ 9.6), whereas those on ring II (p*Ka* ~ 5.6 to ~ 8.0) and ring III (p*Ka* ~ 7.5) have *pKa*'s closer to physiological pH. Gentamicin thus carries a net positive charge under physiological conditions (pH 7.4).

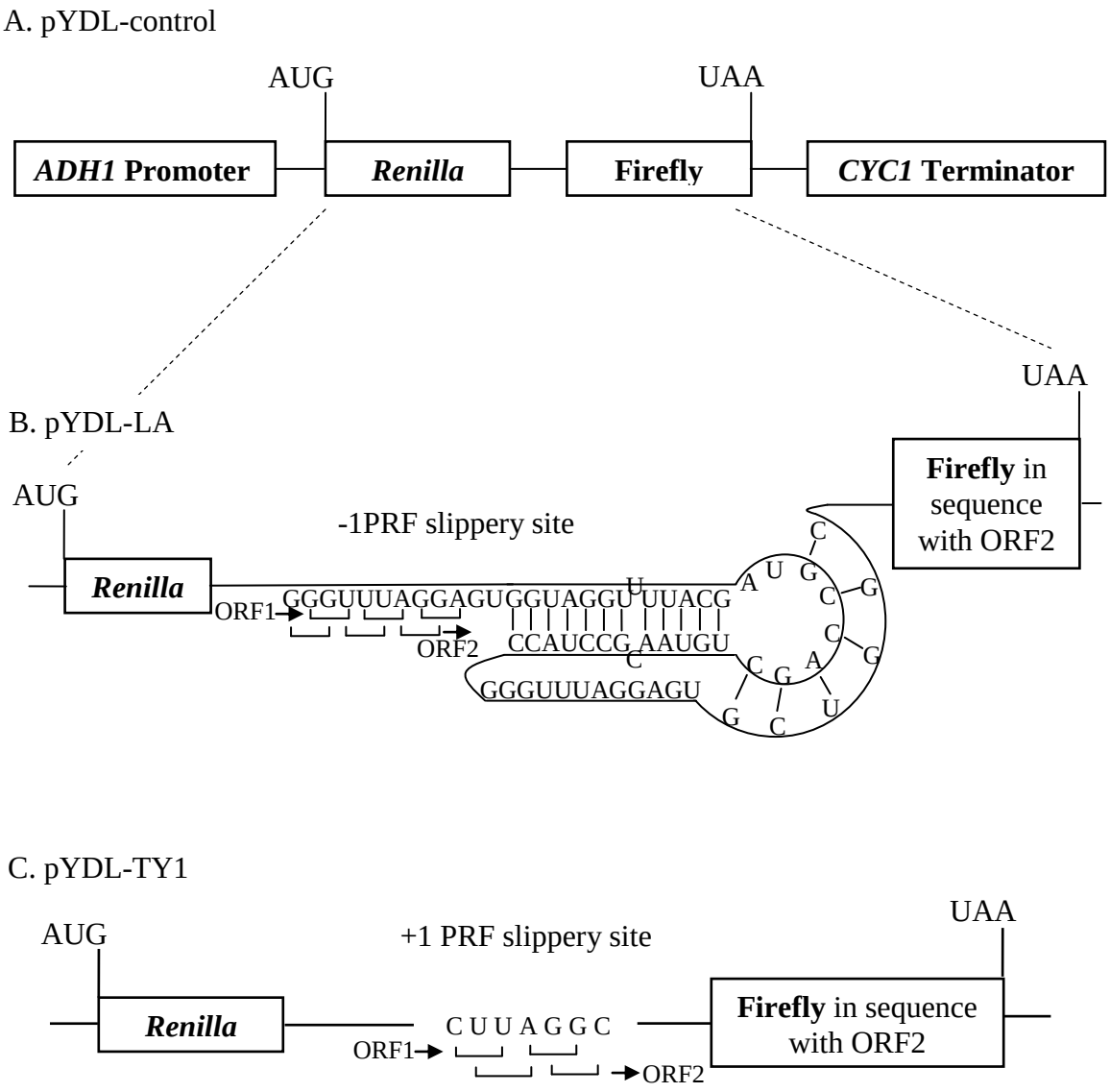


Figure 2. Plasmids used in the *in vivo* dual-luciferase assay. A. pYDL-control was used as control plasmid in this study. **B.** pYDL-LA has a special sequence, X XXY YYZ (the 0-frame is indicated by spaces), which is called a 'slippery site'. An mRNA pseudoknot secondary structure is located immediately 3' to the slippery site, and the pseudoknot could transiently pause the ribosome, and then cause -1 ribosomal frameshifting during translation. **C.** During mRNA translation, the ribosome could slip +1 between CUU and UUA (both Leucine codons). The slippage occurs during a translational pause induced by the slow recognition and low availability of the next in-frame peptidyl-$tRNA_{AGG}$ (Arg). (Modified from Dinman et al., 2006).

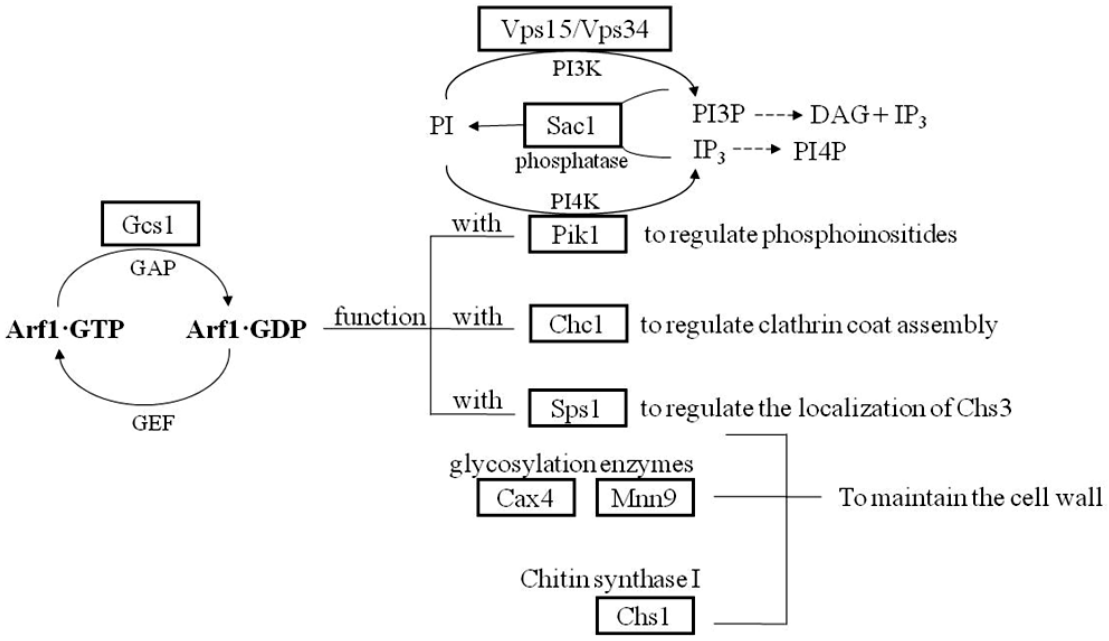
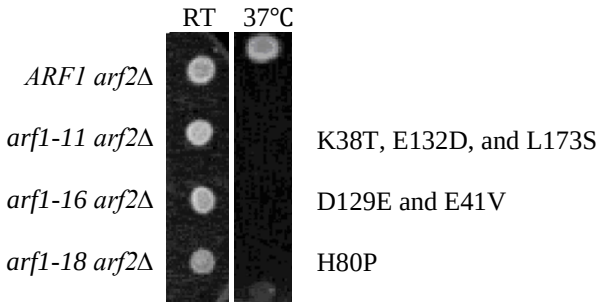


Figure 3. Genetic/Physical network of genes necessary for gentamicin resistance. The single deletion of solid boxed genes is found to exhibit gentamicin sensitivity by Blackburn and Avery (2003).

A.



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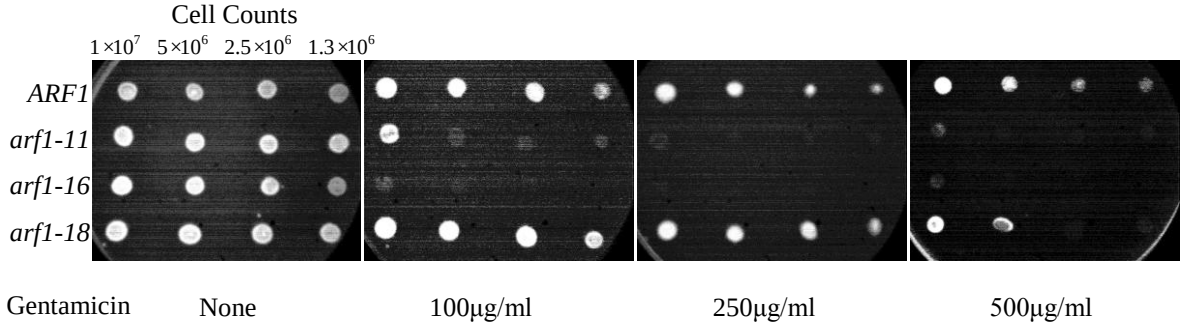


Figure 4. Sensitivity of yeast *ARF1 arf2*Δ and *arf1^{ts} arf1*Δ *arf2*Δ strains to gentamicin. A. *ARF1 arf2*Δ (NYY0-1) and *arf1^{ts} arf1*Δ *arf2*Δ strains (NYY11-2, NYY16-1, or NYY18-1, respectively) (Table 1) were cultured overnight in liquid YPD medium. 30 μl of each overnight culture were transferred to fresh YPD medium plates and grown at their permissive temperature (RT-room temperature) or non-permissive temperature (37 ℃). Images were captured on the second day (for 37 ℃ incubation) or the fourth day (for RT incubation). **B.** Same strains were cultured overnight in liquid YPD medium. A certain number of cells (as indicated) were transferred to fresh YPD medium plates containing different concentrations of gentamicin (none, 100 μg/ml, 250 μg/ml and 500 μg/ml, respectively). The plates were incubated at their permissive temperature (RT) and images were captured on the fourth day.

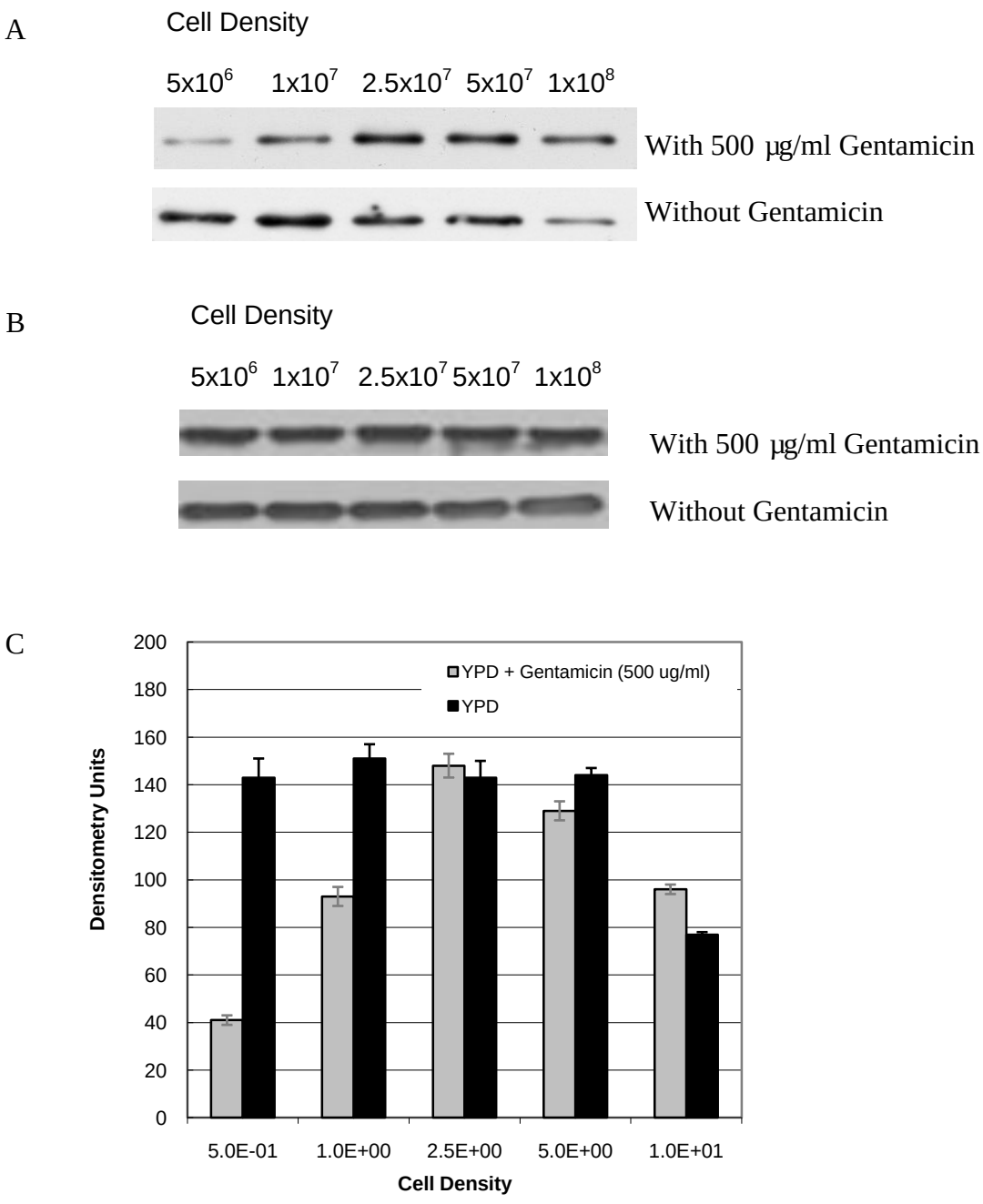


Figure 5. Change in GTP-bound Arf1 levels during different cell growth phases. **A.** GTP-bound Arf1 levels is lower at early-log to mid-log growth phase in the presence of gentamicin (500 µg/ml). **B.** In different growth phases, total Arf1 expression level remains stable, regardless of the presence of gentamicin (500 µg/ml). **C.** Scanning densitometry analysis of Panel **A**.

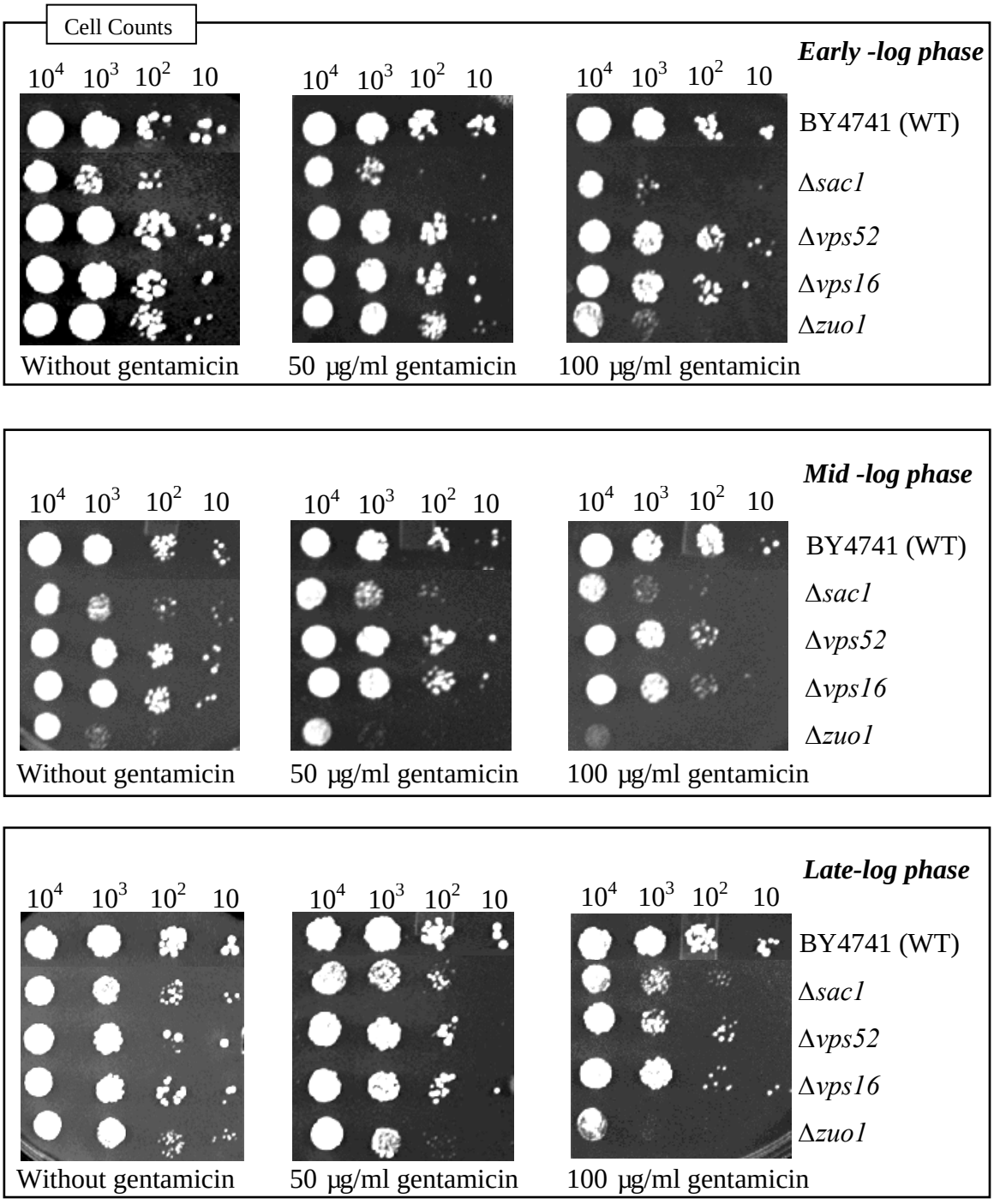


Figure 6. Gentamicin sensitivity of single gene deletion strains in different growth phases. Cells from different growth phases were placed on YPD plates in the absence or presence of gentamicin. *sac1*Δ strain is sensitive to 50 µg/ml of gentamicin; *vps52*Δ strain is sensitive to 50 µg/ml of gentamicin and is more sensitive at mid-log and late-log phases; *vps16*Δ strain is sensitive to 100 µg/ml of gentamicin and is more sensitive at mid-log and late-log phases; and *zuo1*Δ strain is sensitive to 50 µg/ml of gentamicin and is more sensitive at late-log phase.

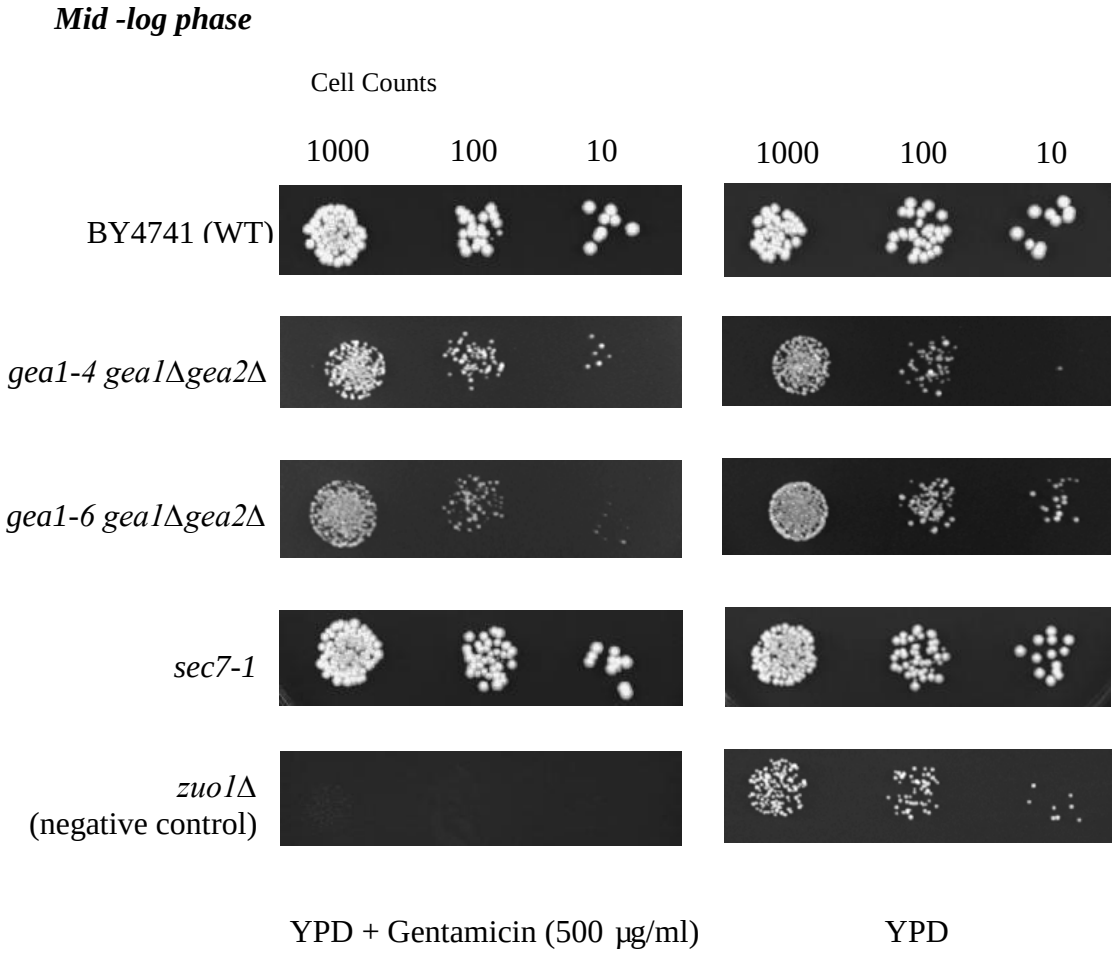


Figure 7. Gentamicin sensitivity of yeast Arf-GEF mutants. *gea1-4 gea1*Δ *gea2*Δ mutant strain has multiple substitutions including two in the highly conserved Sec7 domain (Peyroche et al., 2001). The *gea1-6 gea1*Δ *gea2*Δ mutant has two amino acid substitutions downstream of the Sec7 domain (Peyroche et al., 2001). The *sec7-1* mutant has substitutions outside of the Sec7 domain (Deitz et al., 2000). At its permissive temperature (32 ℃), *gea1-6 gea1*Δ *gea2*Δ cells are sensitive to gentamicin.

Late -log phase

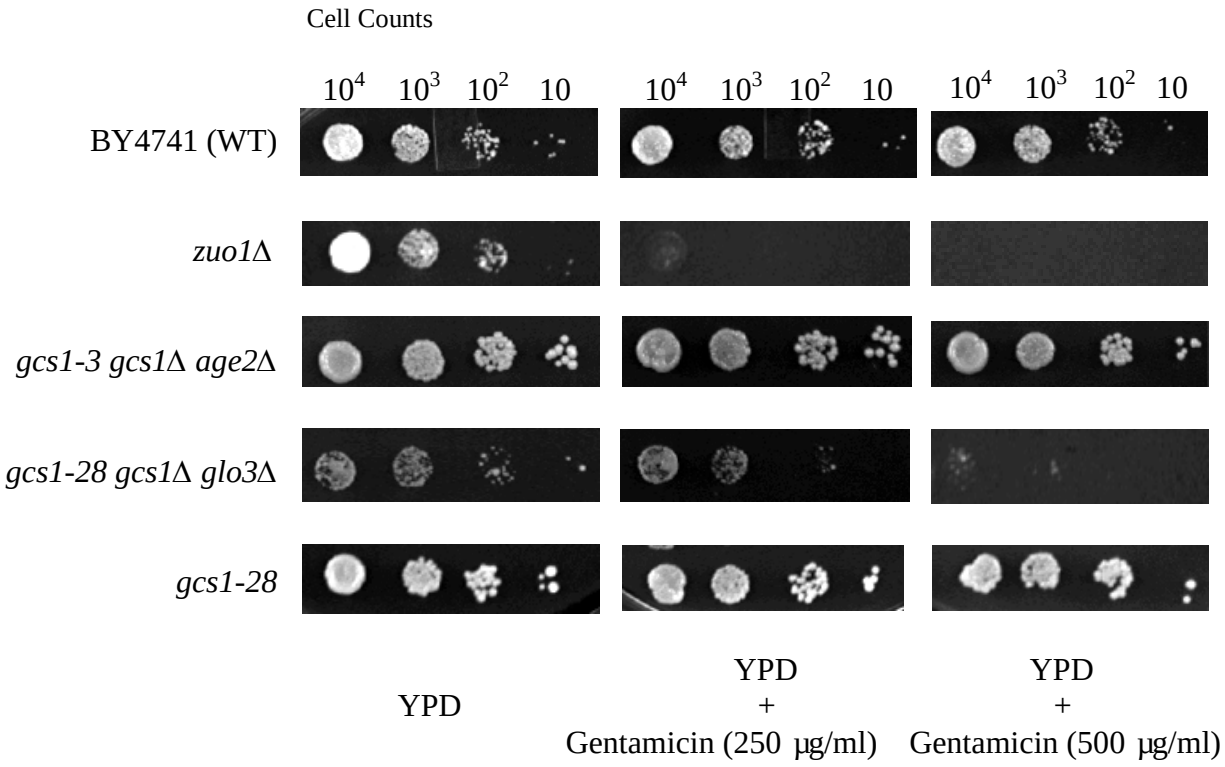


Figure 8. Gentamicin sensitivity of yeast Arf-GAP mutants. Gcs1 and Age2 form an Arf-GAP pair which provides an overlapping function for vesicle transport from the trans-Golgi network to the vacuole and late endosomes (Poon et al., 2001). Gcs1 and Glo3 form another Arf-GAP pair which provides an overlapping function for vesicle retrograde transport from the Golgi to the ER (Poon et al., 1999). The *gcs1-28 gcs1*Δ*glo3*Δ strain showed strong growth inhibition at a gentamicin concentration of 250 µg/ml.

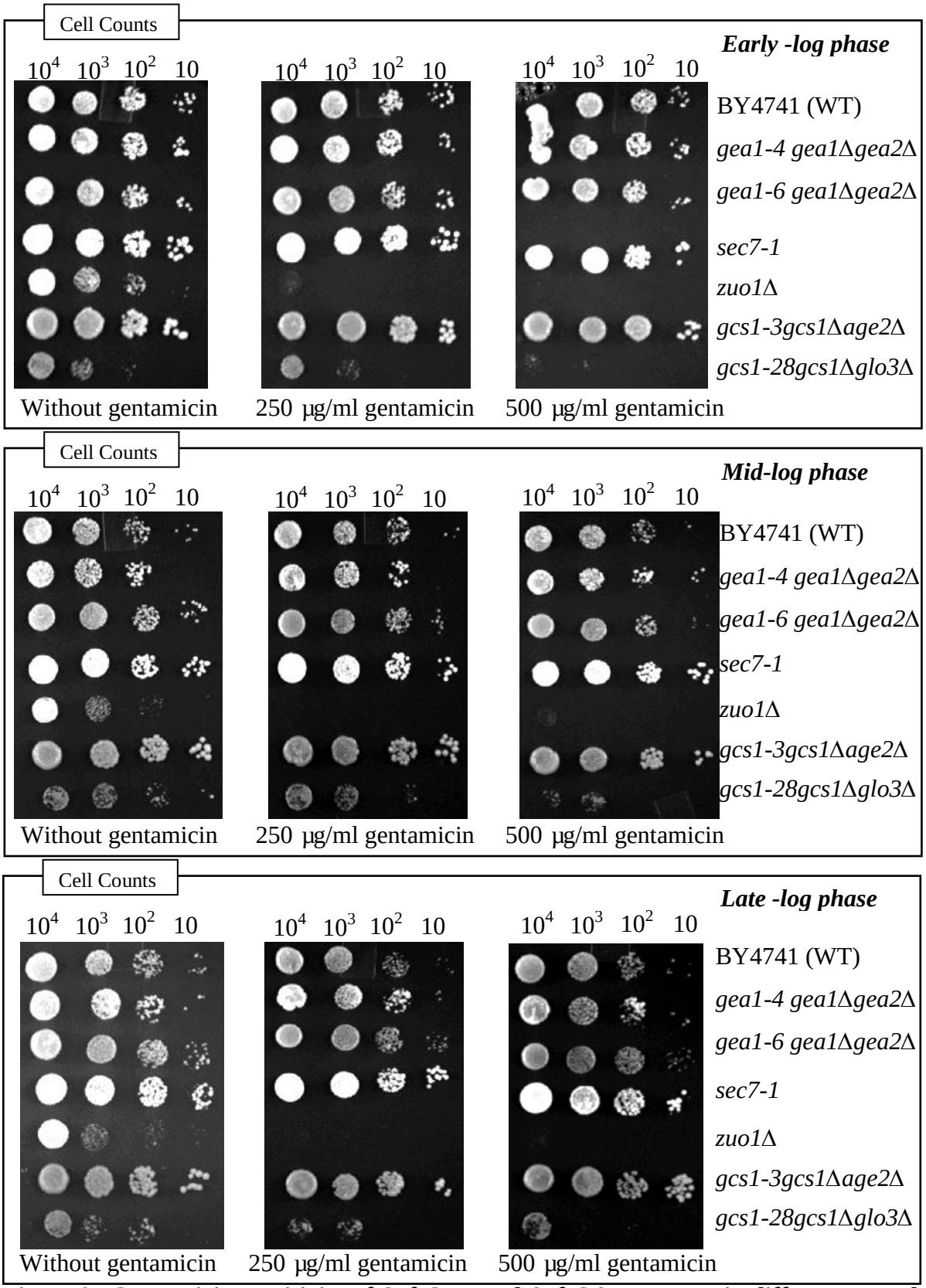


Figure 9. Gentamicin sensitivity of Arf-GEF and Arf-GAP mutants in different growth phases. Cells from different growth phases were placed on YPD plates in the absence or presence of gentamicin at their permissive temperature (30 ℃).

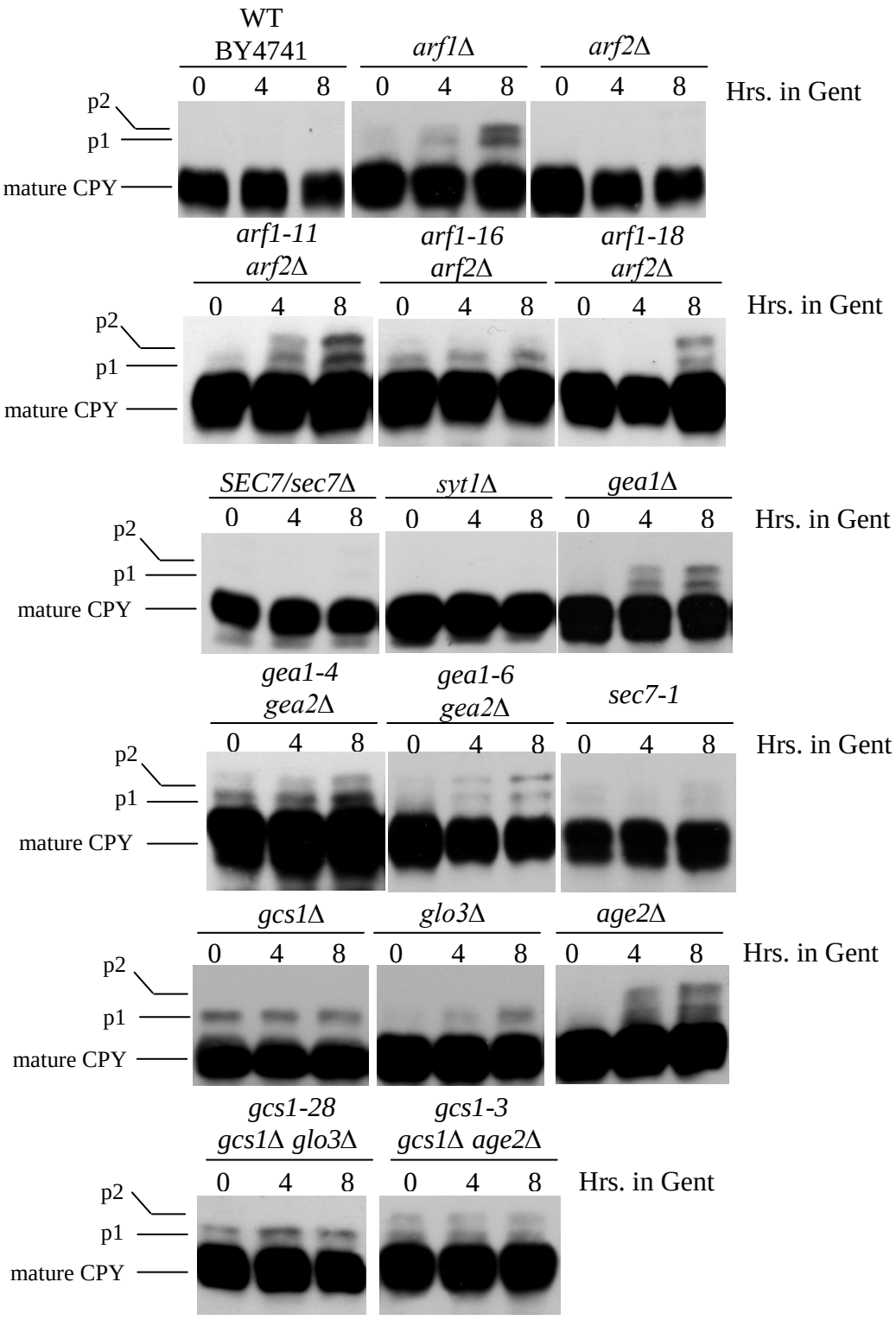


Figure 10. Disruption of cellular CPY processing in Arfs, Arf-GAP, or Arf-GEF mutants by gentamicin. Strains were grown at their permissive temperature to early-log phase (5×10^6 cells/ml) before gentamicin (to a final concentration of 500 μg/ml) was added to the medium. After 0, 4 or 8 hr of additional incubation, aliquots of cells were removed and prepared for Western Blot analysis using anti-CPY as primary antibody.

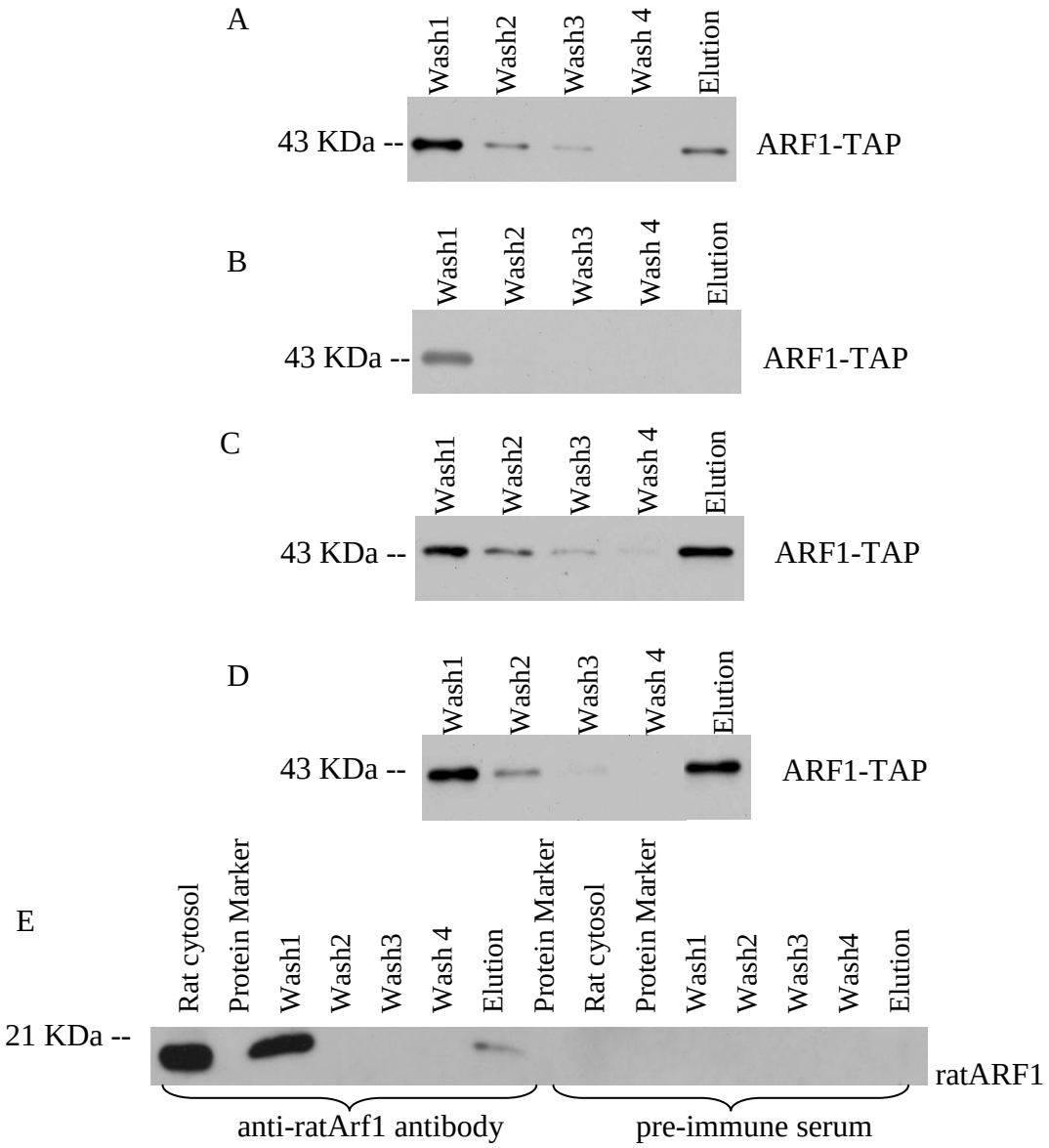


Figure 11. Enrichment of the yArf1-TAP protein and rat Arf1 protein by gentamicin-bound resin. Incubated with yeast Arf1-TAP strain, after four consecutive washes, the elution (by 10 m*M* gentamicin in the lysis buffer) from **A.** gentamicin-bound resin, **B.** empty (negative control) resin, **C.** gentamicin-bound resin with *in vitro* added GDP (10mM) and **D.** gentamicin-bound resin with *in vitro* added GTPγS (10mM). **A** & **B** suggest that gentamicin-bound resin can enrich Arf1-TAP protein *in vitro*. **C** & **D** suggest that Arf1-TAP protein binding to gentamicin-bound resin is enhanced in a GTP/GDP-independent manner. **E.** Rat cytosol was incubated with gentamicin-bound resin. After four consecutive washes, the elution (by 10 m*M* gentamicin in the lysis buffer) was blotted by the rabbit anti-ratArf1 antibody or rabbit pre-immune serum (as negative control).

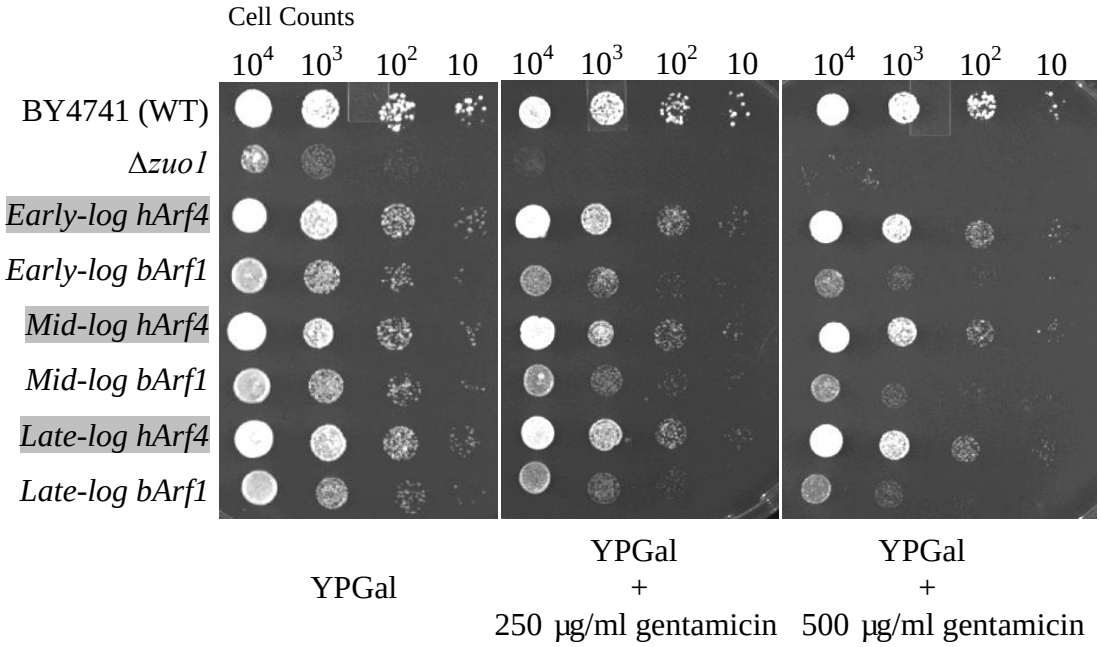
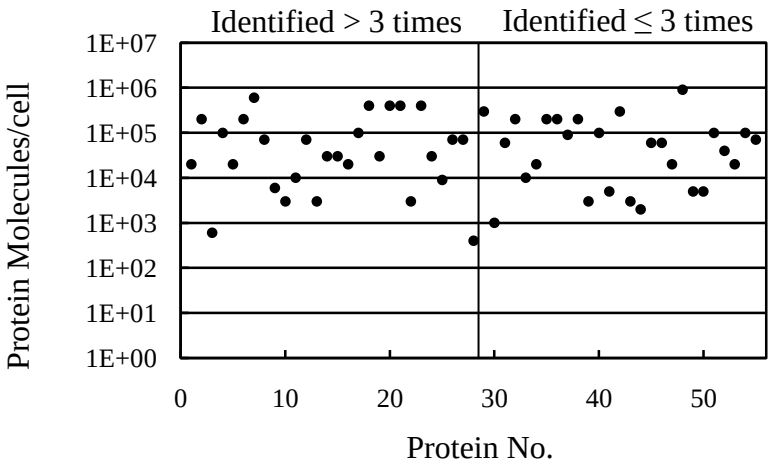


Figure 12. Gentamicin sensitivity of *S. cerevisiae* strains with only hArf4 or bArf1 as functional Arf. Strain hArf4 y*arf1*Δ y*arf2*Δ (RT166 in Table 1) and strain bArf1 y*arf1*Δ y*arf2*Δ (121.13C in Table 1) were cultured at room temperature to early-log, mid-log or late-log phase before the plate spot dilution assays. Media plates were incubated at room temperature and the images were taken on day 3.

A



B

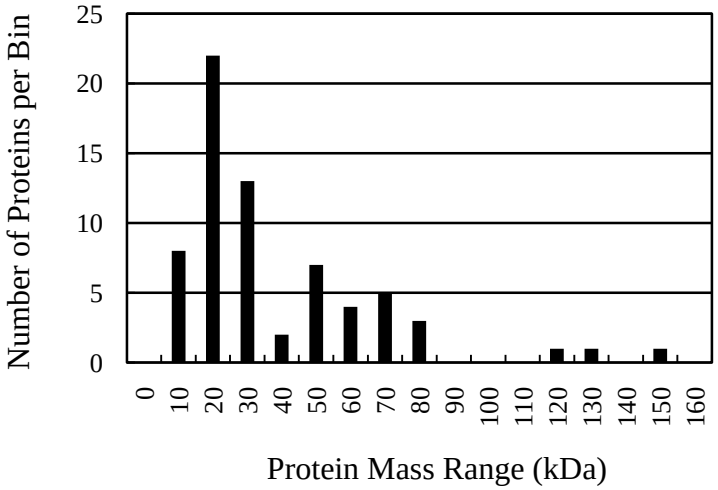


Figure 13. Evaluation of the gentamicin-binding protein list. A. Gentamicin-binding protein expression level. Gentamicin-binding proteins (listed by Protein No. from Table 6) are plotted against their protein expression level (molecules/cell). Protein No.1 to No.28 are detected and identified by LC-MS/MS more than three times. Protein No.29 to No.55 are detected and identified by LC-MS/MS fewer or equal to three times. Protein ID No.56 to No.67 are lack of protein expression data. **B.** Smaller proteins (*Mr* < 35,000) were successfully identified. The limitation of LC-MS/MS in identifying smaller proteins (*Mr* < 35,000) was not observed in our gentamicin-binding protein list. Over half of the proteins identified from this study are smaller proteins (*Mr* < 35,000).

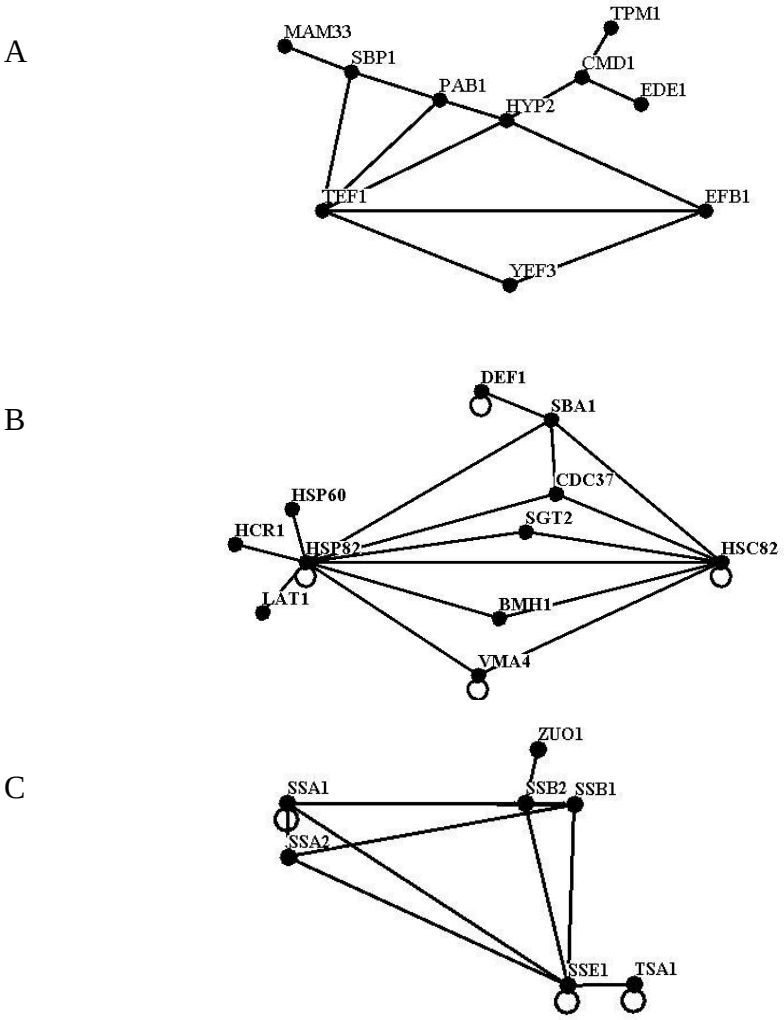


Figure 14. Functional gentamicin-binding protein complexes and protein networks. **A.** Efb1 and Tef1 (identical to Tef2) are members of the eukaryotic elongation factor-1 (eEF-1) complex. The network generated by Osprey revealed eight proteins, Yef3, Hyp2, Pab1, Sbp1, Mam33, Cmd1, Tpm1 and Ede1, that can be eEF-1 binding partners. Also, Abp1 and YKL056C encoding protein were found with Hyp2 and Ede1 in the same protein complex (Costanzo et al., 2010). Bbc1 is in another protein complex with Hyp2 (Gruhler et al., 2002). **B.** Hsc82p and Hsp82p are homologs of GRP94, which has shown the binding ability to gentamicin in a previous study (Horibe et al., 2004). Nine more proteins (Bmh1, Def1, Sba1, Vma4, Hsp60, Hcr1, Lat1, Sgt2 and Cdc37) were grouped as Hsc82 and Hsp82 binding partners. **C.** The ribosome-associated chaperone (RAC) complex. Ssa1, Ssa2, Ssb1 and Sse1 are homologs to HSP73, which has shown the binding ability to gentamicin in a previous study (Miyazaki et al., 2004). Zuo1 is also in our protein list. Based on the Osprey software program, Tsa1 has a protein-protein interaction with Sse1.

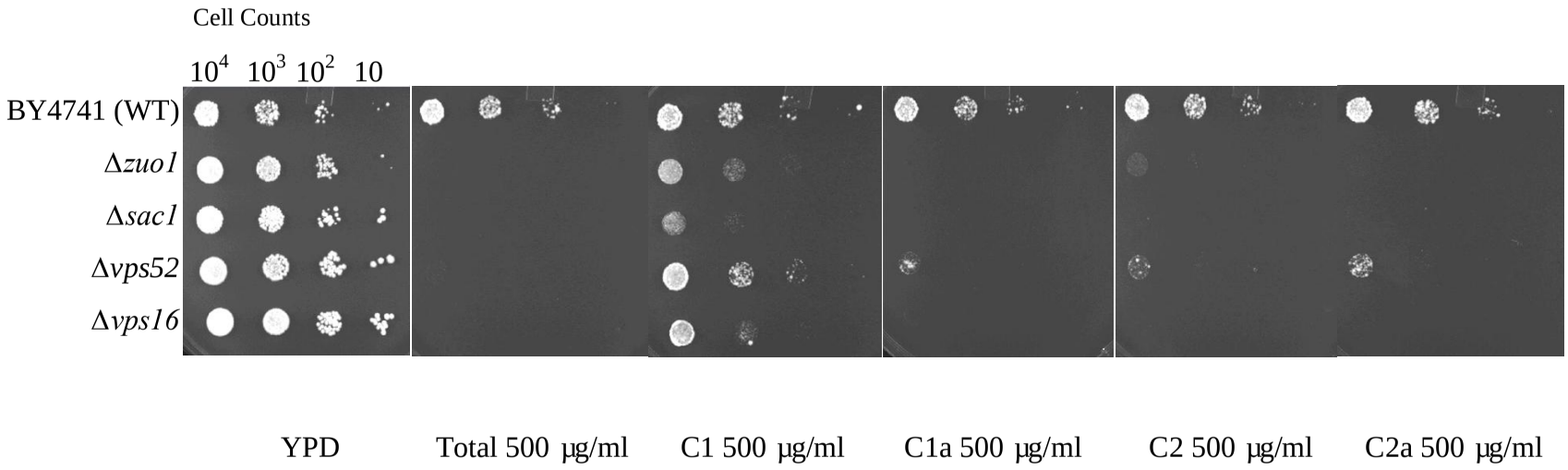


Figure 15. Sensitivity of single gene deletion strains to gentamicin congeners. C1 is the least toxic gentamicin congener.

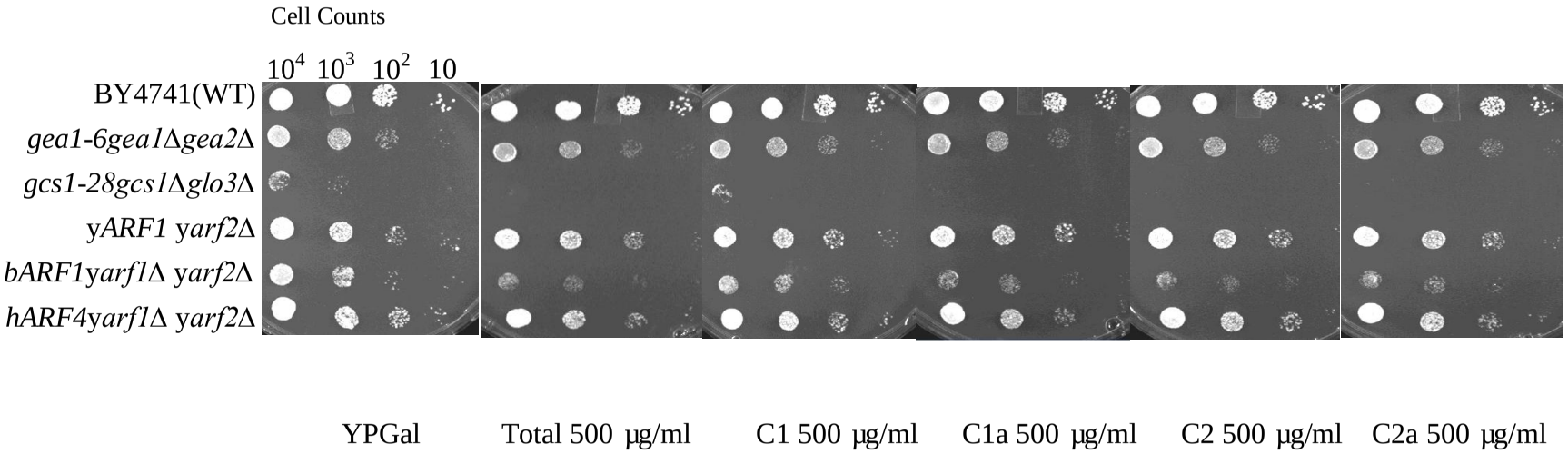


Figure 16. Sensitivity of Arf-GEF mutants, Arf-GAP mutants, and strains with only bArf1 or hArf4 as functional Arf, to gentamicin congeners. C1 is the least toxic gentamicin congener.

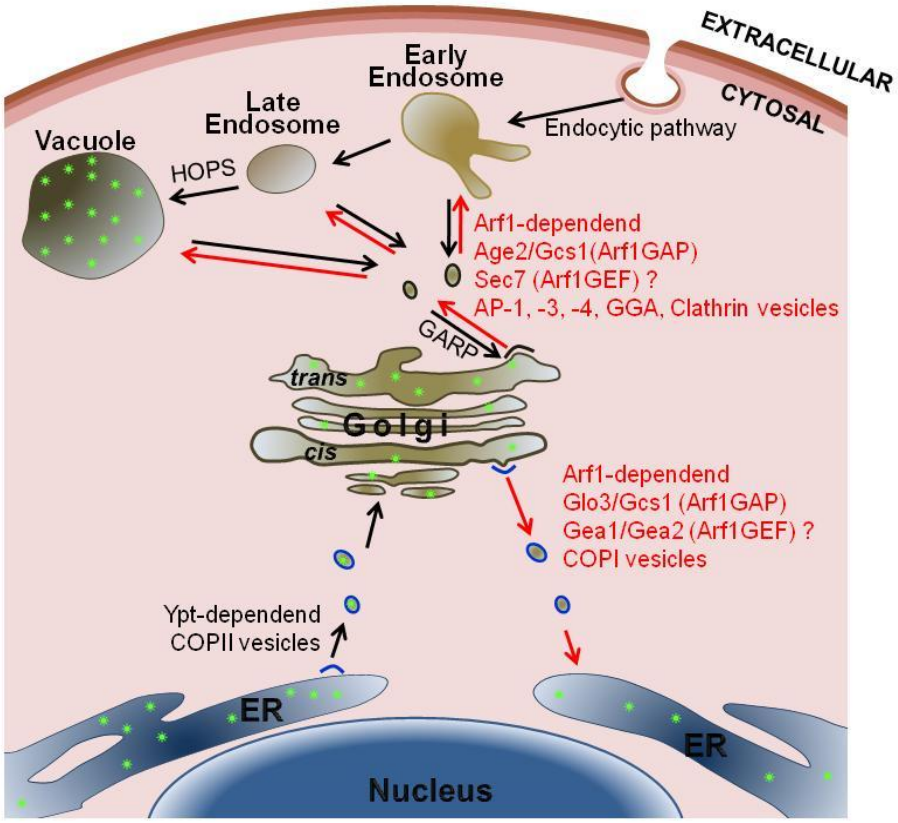


Figure 17. Model of *S. cerevisiae* in response to gentamicin. (, CPY). Arf1 pathway (in red) is a target of gentamicin in eukaryotic cells. Different Arf-GEF and Arf-GAP are responsible for different steps in the anterograde and retrograde traffic. Gentamicin affects the retrograde traffic from the Golgi to the ER significantly. Protein sorting (CPY as a molecular marker) is also affected and blocked by gentamicin as a consequence of the ineffective retrieving of ER lumenal and ER membrane proteins.

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CURRICULUM VITAE

Lin Lin

EDUCATION

2004-2011	Ph.D.	Department of Biochemistry and Molecular Biology Indiana University, Indianapolis, Indiana Major in Biochemistry and Molecular Biology Minor in Microbiology Advisor: Mark Goebl, Ph.D. Dissertation Title: *Gentamicin Induced Intracellular Toxicity in Saccharomyces cerevisiae*
2001-2003	M.S.	Department of Chemistry Tsinghua University, Beijing, China
1997-2001	B.S.	Department of Chemistry Tsinghua University, Beijing, China

WORKING AND INTERNSHIP EXPERIENCE

2004-2011	*Research Assistant* Department of Biochemistry and Molecular Biology Indiana University, Indianapolis, Indiana
2007-2010	*Mentor of Summer Students* Department of Biochemistry and Molecular Biology Indiana University, Indianapolis, Indiana
2003-2004	*Lab Manager and Administrator* Analytical Chemistry Center, Department of Chemistry Tsinghua University, Beijing, China

HONORS AND AWARDS

2009	Third place of the conference paper competition The 13th Great Lakes Area Chinese American Chemical Society
2008	Project SEED summer research program mentor appreciation Indiana section, American Chemical Society
1999-2000	Excellent Academic Achievement Award Tsinghua University, China

PUBLICATIONS

1. Lin L., Wagner, M. C., Cocklin, R., Kuzma, A., Harrington, M. A., Molitoris, B. A., & Goebl, M. G. (2011). The antibiotic gentamicin inhibits specific protein trafficking functions of the ARF1/2 family of GTPases, *Antimicrobial Agents and Chemotherapy*, *55* (1), 246-254.

2. Lin L., Chan, S., Zhao, F., Tang, Y., & Zhang, F. (2004). Study of nicotine acting as an uncompetitive inhibitor of lipase, *Acta Chimica Sinica*, *62* (10), 1003-1006.

3. Lin L., Tang, Y., Zhang, F., Zhou, J., & Hu, S. (2003). Using chemical methods to crosslink xenogeneic heart valves: the progress of bioprosthetic heart valves, *Acta Academiae Medicinae Sinicae*, *25* (6), 735-737.

4. Lin L., Tang, Y., Liang, Q., Zhou, J., Cui, J., Hu S., & Zhang, F. (2003). Crosslinking bovine pericardial bioprosthetic heart valves by phthalocyanine sensitized photooxidation reaction, *Acta Academiae Medicinae Sinicae*, *25* (6), 671-675.